HUMAN LUTEINIZING HORMONE

STRUCTURE AND FUNCTION OF SOME PREPARATIONS

J. G. LOEBER

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Proefschrift

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE WISKUNDE EN NATUURWETENSCHAPPEN AAN DE KATHOLIEKE UNIVERSITEIT TE NIJMEGEN OP GEZAG VAN DE RECTOR MAGNIFICUS PROF. DR. A.J.M. VENDRIK, VOLGENS BESLUIT VAN HET COLLEGE VAN DECANEN IN HET OPENBAAR TE VERDEDIGEN OP 22 APRIL 1977 DES NAMIDDAGS TE 4.00 UUR

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KRIPS REPRO - MEPPEL

Never measure the height of a mountain
until you have reached the top.
Then you will see how low it was.
(Dag Hammarskjöld, *Markings*)

Het in dit proefschrift beschreven onderzoek werd begeleid door Dr. R.M. Lequin en uitgevoerd in het RIA laboratorium van de Kliniek voor Gynaecologie en Verloskunde (hoofden: Prof.Dr. T.K.A.B. Eskes, Prof.Dr. J.L. Mastboom) van de Katholieke Universiteit te Nijmegen.
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INDEX

LIST OF ABBREVIATIONS

ACTH - Adrenocorticotropic hormone

ATP - Adenosine triphosphate

cAMP - Cyclic 3',5'-Adenosine monophosphate

CG - Chorionic gonadotropin

FSH - Follicle-stimulating hormone (Follitropin)

GH - Growth hormone

LH - Luteinizing hormone (Lutropin)

PMS - Pregnant mares' serum gonadotropin

TSH - Thyroid-stimulating hormone (Thyrotropin)

1^{st} IRP LH/FSH bio - First International Reference Preparation for bioassay of LH and FSH

1^{st} IRP LH imm - First International Reference Preparation for immunoassay of LH

1^{st} IRP TSH imm - First International Reference Preparation for immunoassay of TSH

2^{nd} IRP hMG - Second International Reference Preparation for human Menopausal Gonadotropin

The purpose of the investigation reported in this thesis, was to elucidate structural and functional properties of human luteinizing hormone.

Luteinizing hormone (LH) is one of the many hormones secreted by the pituitary gland. Together with follicle stimulating hormone (FSH), also produced by the pituitary gland, and chorionic gonadotropin (CG) produced by the placenta, it forms the group of the gonadotropic hormones. From a chemical point of view these hormones show great resemblance. They consist of a protein part as well as a carbohydrate moiety; hence, they are called glycoproteins. A third pituitary glycoprotein hormone is thyroid stimulating hormone (TSH).

A special feature of these four hormones is that they consist of two structurally dissimilar subunits. Within a particular species one of these, the α-subunit is more or less identical in all four with respect to amino acid composition and primary structure of the protein part. The other, the β-subunit, varies and is believed to be the hormone-specific subunit. In mammals, the main function of the pituitary gonadotropins FSH and LH is to regulate the reproductive function. More specifically, they control the gonadal activity. In the female FSH promotes growth of the ovarian follicles, while LH causes ovulation and stimulates luteinization of the follicular cells. In the male, the target for FSH are the Sertoli cells through which

spermatogenesis in the germinal epithelium is initiated; LH stimulates the Leydig cells to the production of androgens. However, FSH and LH often are considered to be a "gonadotropic hormone complex" for the following reasons. (1) By chemical means it is very difficult to separate the one from the other; usually one regards the purified preparations of the one hormone as being "contaminated" by the other. (2) Functionally, they act in concert to bring about the many facets of what is called "gonadal function", especially in the control of the gonad in the female.

Notwithstanding the above mentioned complexity, conventional hormone research has recognized differing functions for these two hormones. As a consequence of these known functions bioassays were developed. A bioassay is a functional assay, assessing the relative potency of a hormone on the basis of its known qualitative effects. Such an assay may employ living animals (*in vivo* bioassay) or isolated target tissues (*in vitro* bioassay). It has been recognized that the *in vitro* bioassay has several advantages when compared with the *in vivo* bioassay, e.g. it is less cumbersome, shows a greater reproducibility, as well as greater sensitivity. Yet, search for new assay methods persisted, in particular with the objective to further increase the sensitivity. When it was discovered that peptide hormones are also immunologically active, assays based upon this type of structural activity were developed. In the late 1950's the radioimmunoassay (RIA) was introduced by

R.S. Yalow and S.A. Berson. This method was a thousand-fold more sensitive than the conventional *in vivo* assay methods and moreover was technically relatively easy to perform. The RIA is nowadays one of the routine methods of choice, for example for clinical purposes. There is, however, a fundamental difference between the bioassay and the RIA. Bioassays measure the function of a hormone, whereas in the RIA the concentration of the hormone itself is measured. It has to be kept in mind that for many protein hormones it is not yet clear to what extent the biological (B) and immunological (I) activities are related. For the time being, therefore, B and I have to be regarded as independent characteristics of a hormone. In the last decade a type of assay has been developed, which is more or less a hybrid between the RIA and the *in vitro* bioassay, namely the radioligand assay (RLA). In this assay the hormone is not bound to antibodies but to membranes of cells of the target organ. Details of these various assays are presented in the pertinent chapters of this thesis.

Apart from the fact that knowledge of human gonadotropins has intrinsic scientific value, clinical application of such hormones requires the use of highly purified *human* preparations, as comparative studies concerning gonadotropic hormones in other vertebrates revealed considerable variability in structure and function. Although in the past decade a large number of human LH preparations have been made, our knowledge of the properties

of this hormone is still limited. This situation prompted us to undertake an investigation concerning the characteristics of human luteinizing hormone. It involved extraction and isolation as well as identification of the hormone structure and, more importantly, an analysis of its biological and immunological properties.

To carry out these studies, a sufficient amount of LH was needed. Human pituitary material is very scarce and, if at all commercially available, very expensive. Thanks to the cooperation of the Dutch Growth Foundation and through the courtesy of Dr. M. Zachmann (Kinderspital, Zürich, Switzerland) we obtained several batches of crude pituitary material containing the glycoprotein hormone fraction. In close collaboration with the research group of Dr. G. Hennen (Hôpital de Bavière, Liège, Belgium) highly purified LH preparations were made.

The purification procedure is described in chapter 2. The characterization of the preparations employing several assay methods is the subject of chapters 3 - 8. In chapter 9 the results obtained with the various methods are compared and discussed.

Part of the results has been published elsewhere *(Loeber & Lequin, 1975, Loeber, Van Doornenberger & Lequin, 1977)*.

2.1. Introduction

Human pituitary glands usually are collected at autopsy 24 - 48 hours post-mortem and preserved either dehydrated after acetone treatment, or frozen. At least six hormones are fairly easy to isolate, namely growth hormone (GH), adrenocorticotropic hormone (ACTH), prolactin and the glycoproteins LH, FSH and TSH. Irrespective of the way of preservation an initial separation of the glycoproteins from the other three hormones can be achieved by making use of the difference of their solubility in alcohol or in salt-saturated solvents. *Koenig & King (1950)* developed a method of extracting acetone-dried glands with a mixture of 1.3 M ammonium acetate buffer pH 5.1 and alcohol (60:40 v/v) in which only the glycoproteins are soluble. From the residue the other hormones can then still be extracted. Frozen glands are commonly treated with 0.01 M phosphate buffer pH 6.2 *(Roos & Gemzell, 1964; Roos, 1967)*, whereby all hormones are extracted; isolation of the glycoproteins can then be achieved by fractionated ammonium sulphate precipitation *(Squire & Li, 1959; Ward, Adams-Mayne & Wade, 1961; Rathnam & Saxena, 1970)*. The next step is the separation of the glycoproteins from one another. The easiest and most widely used method is chromatography on ion-exchange resins, e.g. the carboxymethyl-(CM-) or diethylaminoethyl-(DEAE-)derivatives of cellulose or Sephadex, Amberlite CG 50 etc. *(Squire & Li, 1959; Steelman & Segaloff, 1959; Ward,*

McGregor & Griffin, 1959; Woods & Simpson, 1961, Ward et al, 1961; Reichert & Parlow, 1964a; Hartree, Butt & Parkhar, 1964; Rathnam & Saxena, 1970; Torjesen, Sand, Norman, Trygstad & Ross, 1974, Sairam & Li, 1975; Roos, Nyberg, Wide & Gemzell, 1975; Closset, Van Dalem, Hennen & Lequin, 1975). Methods for further purification comprise preparative electrophoresis on polyacrylamide gel or starch gel *(Li, 1958; Butt, Crooke, Cunningham & Wolf, 1963; Roos & Gemzell, 1964)*, gelfiltration on G 100 or G 200 *(Bettendorf, Apostolakis & Voigt, 1969; Reichert & Parlow, 1965; Rathnam & Saxena, 1970)* and chromatography on hydroxylapatite *(Butt, Crooke & Cunningham, 1961).*

It cannot be stated which method is to be preferred, as each one yields more or less "pure" hormone preparations. The purification schedules used for the project described in this thesis are largely those according to *Closset et al (1975).*

2.2. Materials and methods

Crude glycoprotein material from acetone-dried pituitary glands was made available by the Dutch Growth Foundation. Crude glycoprotein material from frozen pituitary glands was a gift from Dr. M. Zachmann (Zürich, Switzerland). All chemicals were reagent grade mainly from Merck (Darmstadt, Germany). Sephadex ion-exchange resins and filtration gels were obtained from Pharmacia (Uppsala, Sweden) and treated as prescribed by the manufacturer. To obtain the protein elution profile of the columns, the absorbancy of the effluent at 235 or 280 nm was measured with a Beckman Dual Beam spectrophotometer, or the protein concentration in aliquots of

the columnfractions was determined according to *(Miller (1959)*. To measure the protein content of hormone preparations more precisely the method of *Lowry, Rosebrough, Farr & Randall (1951)* was used with Bovine Serum Albumin (BSA, Sigma) as a standard. Fraction volumes were reduced by ultrafiltration in Amicon cells supplied with a UM-2 filter.

2.3. Procedure (fig. 2-1)

2.3.1. Separation of LH_I and FSH from LH_{II} and TSH

Crude glycoprotein material from either acetone-dried or frozen glands was dissolved in 0.005 M Na-phosphate buffer pH 6.2. Any insoluble material was removed by centrifugation. The clear brown solution was applied on a CM-Sephadex C 25 column (30 x 5 cm), previously equilibrated in the same buffer. The brown-yellow non adsorbed fraction was collected and the column washed till the absorbancy of the effluent at 235 nm (A_{235}) was lower than 0.05 (fraction C_I). Next, the column was stripped by 0.5 M NaCl dissolved in buffer and washed till the A_{235} of the effluent was lower than 0.05 (fraction C_{II}).

2.3.2. Fractionation of C_I; separation of LH_I and FSH

Solid $(NH_4)_2SO_4$ was added to fraction C_I till 80% saturation. The solution was left overnight. The next day the precipitate was collected by centrifugation (20 min at 15000 g) and

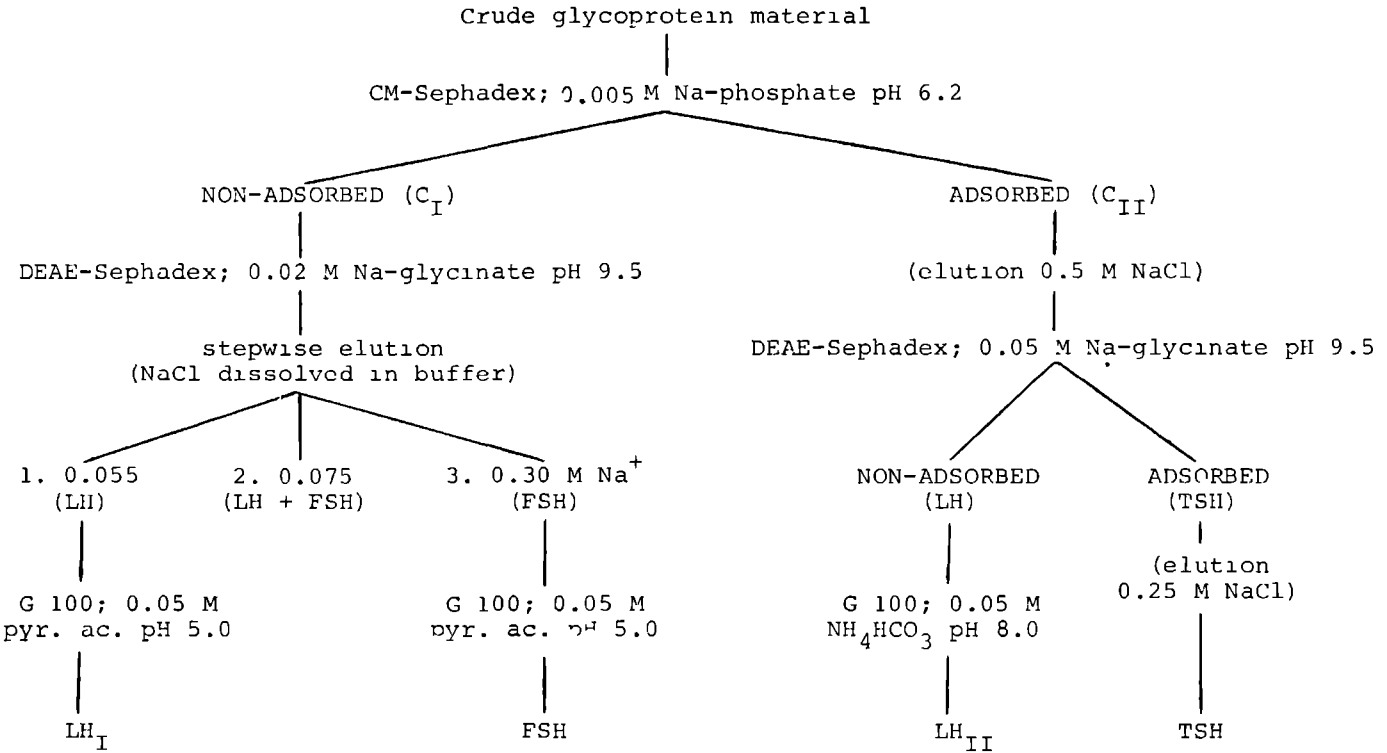

Fig. 2-1. Extraction and purification of human glycoprotein hormones from pituitary material.

dissolved in 50 ml of distilled water while the supernatant was discarded. The solution was desalted by gelfiltration on G 25, equilibrated in 0.02 M Na-glycinate buffer pH 9.5 and applied on a DEAE-Sephadex A 25 column (20 x 5 cm) equilibrated in the same buffer. Under these conditions most of the LH and FSH will adhere to the column. The LH, however, can be eluted with low ionic strength. To assess which ionic strength or molarity of the eluting buffer is optimal for separation of the two hormones a pilot study was performed in which the DEAE-column was eluted stepwise with small increments in buffer molarity. The increments were made by adding NaCl to the buffer each time after A_{235} of the effluent was lower than 0.05. By means of specific radioimmunoassay systems *(chapter 7)* the amounts of FSH and LH in each fraction were measured (fig. 2-2). It appeared to be impossible to achieve a complete separation of the two hormones. However, the pilot study indicated that it was possible to obtain relatively pure preparations at the expense of losing some material in a mixed hormone fraction: elution with 0.055 M Na^+ removed most of the LH activity with a minimal amount of FSH activity, increasing the molarity of Na^+ to 0.075 M yielded a fraction with both LH and FSH activity, while the fraction obtained after having increased the Na^+ concentration to 0.3 M contained little LH and much FSH activity. The three fractions thus obtained (C_IA 0.055, C_IA 0.075, C_IA 0.3) were concentrated in an Amicon ultrafiltration apparatus. The fraction C_IA 0.075 was stored at -20°C, the other two were further purified as described

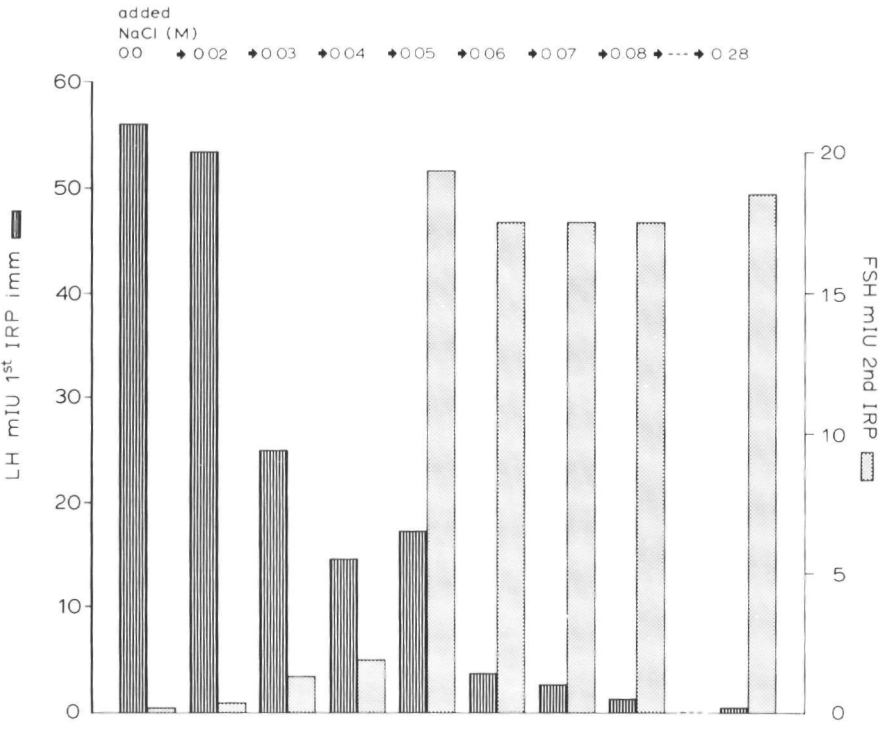

Fig. 2-2. Stepwise elution of C_I, adsorbed on DEAE-Sephadex A 25 in 0.02 M Na-glycinate pH 9.5, by addition of increasing amounts of NaCl. LH and FSH activities were measured by radioimmunoassay.

below.

2.3.2.1. Purification of C_IA 0.055

This fraction was further purified by ascending gelfiltration on G 100 (300 x 5 cm) in 0.05 M pyridine acetate pH 5.0. The elution profile was monitored both by protein determination according to *Miller (1959)* and by radioimmunoassay. The column fractions containing LH activity were pooled; this pool was

concentrated by Amicon ultrafiltration and either stored at -20°C or lyophilized. LH preparations obtained by this procedure will be further referred to as LH_I.

2.3.2.2. Purification of C_IA 0.3

This fraction was purified as described for C_IA 0.055. The column fractions containing FSH activity were pooled; this pool was concentrated by Amicon ultrafiltration and stored at -20°C. (Further purification was performed by Closset and his co-workers in Liège, Belgium, and will not be described in this thesis).

2.3.3. Fractionation of C_{II}; separation of LH_{II} and TSH

Solid $(NH_4)_2SO_4$ was added to fraction C_{II} till 80% saturation. The solution was left overnight. The next day the precipitate was collected by centrifugation (20 min at 15000 g) and dissolved in 50 ml of distilled water, while the supernatant was discarded. The solution was desalted by gelfiltration on G 25 equilibrated in 0.05 M Na-glycinate buffer pH 9.5 and applied to a DEAE-Sephadex A 25 column (20 x 5 cm), equilibrated in the same buffer. The non adsorbed fraction, which is mainly LH, was collected and the column washed till the A_{235} of the effluent was lower than 0.05. The total eluate (C_{II}A 0.05) was concentrated by Amicon ultrafiltration and further purified as described below. The molarity of the

eluting buffer was then increased to 0.25 by addition of NaCl and the column eluted till A_{235} lower than 0.05. This eluate, which is mainly TSH, was concentrated by Amicon ultrafiltration and stored at -20°C. (Further purification was performed by Closset and his co-workers in Liège, Belgium *(Closset, 1975)* and will not be described in this thesis)

2.3.3.1. Purification of $C_{II}A$ 0.05

This fraction was further purified by gelfiltration on G 100 (100 x 5 cm) in 0.05 M NH_4HCO_3 pH 8.0. The elution profile was monitored both by reading absorbancy at 280 nm and by radioimmunoassay. The fractions containing LH activity were pooled and lyophilized. LH preparations obtained by this procedure will be further referred to as LH_{II} preparations.

2.3.4. Preparation of LH subunits

About 50 mg of a purified LH_I or LH_{II} preparation was dissolved in 10 ml of a solution of 8 M urea in 0.05 M HCl pH 2.8. The solution was incubated at room temperature for 16 - 20 h. The pH of the solution was adjusted to 9.5 with 1 N NaOH and diluted with 40 ml of 0.01 M Na-glycinate pH 9.5. The solution was then quickly applied to a small DEAE-Sephadex A 25 column (2 x 10 cm) previously equilibrated in the same buffer. The β-subunit, which was not adsorbed, was collected by washing the column. The α-subunit was eluted by

application of a lineair gradient of NaCl dissolved in the glycinate buffer and ranging from 0 - 0.5 M. Both subunit fractions were desalted on G 25, equilibrated in 0.05 M NH_4HCO_3 pH 8.0 and lyophilized. Each subunit was submitted to the same treatment once more to remove contaminations of the other subunit as well as some undissociated LH. Finally, they were gelfiltrated on G 100 (2 x 100 cm) in 0.05 M NH_4HCO_3 pH 8.0 and lyophilized.

2.4. Results and discussion

The described purification procedure was performed eight times, six times with material from acetone-dried glands yielding the batches coded NM 07, 08, 09, 14, 15, 16 and twice with material from frozen glands yielding the batches coded NM 10 and 11. Good separation of major quantities of FSH and LH was achieved in all cases. However, the presence, after the first chromatography, of a substantial amount of LH in the crude FSH-fraction may not be surprising in view of similar results reported by other investigators. Usually such LH contamination of FSH has been destroyed in the process of FSH purification by treatment with chymotrypsin *(Reichert, 1967)* or urea *(Saxena & Rathnam, 1968)*, partitioning *(Butt, Crooke & Wolf, 1965)* and electrophoresis *(Graesslin, Weise & Bettendorf, 1972)*. Because the conditions for separation of LH and FSH during fractionation of C_I on the DEAE-Sephadex A 25

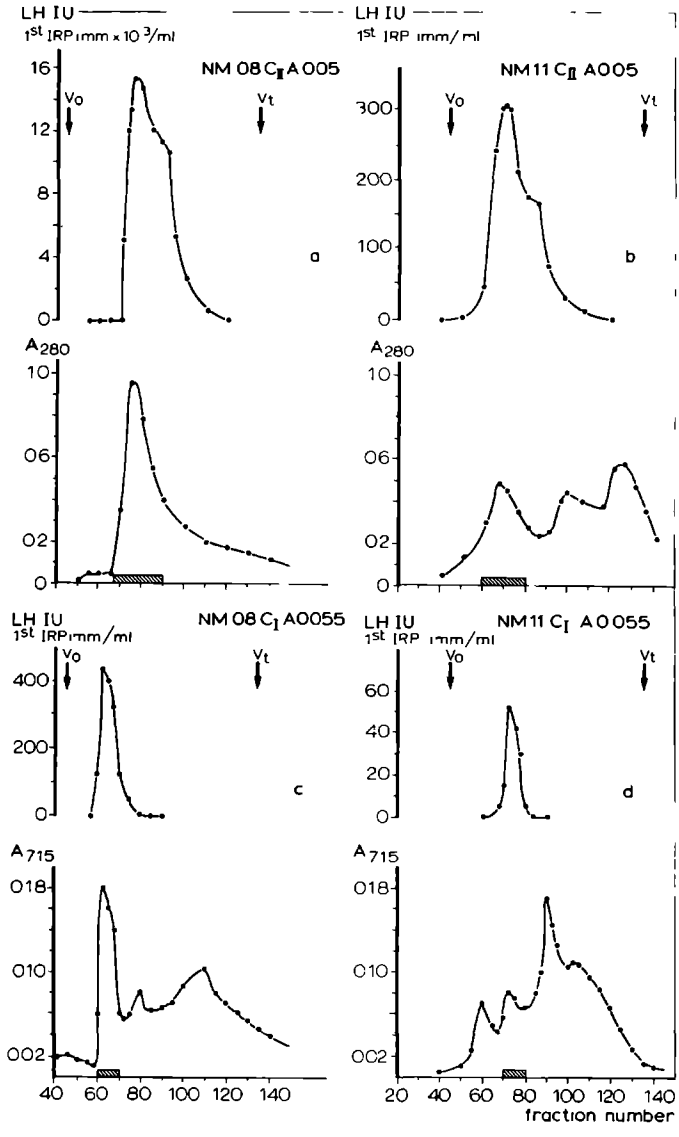

Fig. 2-3. Elution profiles of hLH fractions after final gel-filtration on G 100: a. NM 08 C_{II} A 0.05, b. NM 11 C_{II} A 0.05, c. NM 08 C_{I} A 0.055, d. NM 11 C_{I} A 0.055. Solid lines indicate protein as determined by reading A_{280} (a,b) or A_{715} according to Miller (c,d). Broken lines indicate LH activity as determined by radioimmunoassay. Hatched bars indicate fractions pooled for further use. V_o = void volume, V_t = total volume.

column were unknown, a pilot study was carried out as described in section 2.3.2. To be able to evaluate the results in a short time specific radioimmunoassays for LH and FSH were used in a first instance. By means of *in vivo* bioassay techniques *(chapter 5)*, however, the separation, based upon the results of these RIA's, was confirmed: the final LH_I and FSH preparations were minimally contaminated by each other. The success of isolating this particular LH, called LH_I, which differed from the major fraction of LH (LH_{II}), points up the question whether these two LH forms reflect the existence of so-called micro-heterogeneity. This interesting problem will be discussed in chapter 9.

The major drawback of using starting material extracted from frozen glands compared with that from acetone-dried glands is that the former contains a larger amount of inert protein including blood pigments, which are difficult to eliminate. Fortunately, because these proteins behave similar to FSH on the ion-exchange columns, at least the LH preparations are relatively free of them. However,if the final G 100 elution profiles are compared (e.g. LH_{II} from NM 08 and NM 11, fig. 2-3a,b) it is clear that it cannot be decided from the protein profile alone in which peak the LH activity resides. Once the behaviour of LH on G 100 gelfiltration has been established by precise determination of K_{av}-values *(chapter 3)* this information could be of use in subsequent purification procedures. Another possibility is to measure LH activity by radioimmunoassay to distinguish between the

Table 2 - I. Data concerning human pituitary preparations and results of LH purification.[1)]

	Starting material			LH_I			LH_{II}		
Batch[2)]	Initial wt (g)	Approx. no of pit. glands	Spec.biol. act. (IU 1^{st}IRP LH bio/mg	Final wt (mg)	Spec.biol. act. (IU 1^{st}IRP LH bio/mg	Purif. factor	Final wt (mg)	Spec.biol. act. (IU 1^{st}IRP LH bio/mg	Purif. factor
NM 07	5.2	2600	260	16	13700	55	58	6050	23
08	12.2	7600	240				205	1490	6
09	8.1	8600	400	47	7900	20	255	4400	11
14	14.0	7600	260	72	4700	29	240	8400	42
15	10.0	7000	195	13	9450	48	96	12500	64
16	17.0	5000	210	66	3060	15	266	9550	45
10	13.5	2800	165	10	5100	31	30	4580	28
11	12.5	2600	165	26	2940	18	36	3700	22

1) For definition of terms, see text.
2) NM 07 - NM 16: acetone-dried glands; NM 10 - NM 11: frozen glands.

hormone and other proteins. This technique has been used here. The radioimmunoassay system utilises an antiserum directed towards LH_{β}-subunits. LH preparations always contain a certain amount of free subunits which are partially separated from the intact hormone by gelfiltration on G 100, due to the difference in molecular size. The shoulder in the descending part of the peak concerning the immunologically active LH represents the β-subunit contamination. A similar situation holds for LH_I preparations (Fig. 2 - 3c,d).

In Table 2-I the quantities of starting material as well as the yields in terms of dry weight of both types of LH for the various batches are compiled.[1)] Although these figures give an indication of the efficiency of this schedule, when a purification procedure has to be judged for its merits not only the quantity of the products has to be taken into account, but also the quality. In the case of hormones the quality can be correlated to the specific biological activity, i.e. the biological activity per unit weight. It is difficult to compare the absolute figures for the biological activities attained by one's own procedure with those reported in the literature due to variability in bioassays as well as in standards and reference preparations used. It is possible, however, to state the "purification factor", i.e. the ratio of the specific biological activities of the end-product and the starting material (Table 2-I). Unfortunately few investigators report these factors.

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1) The LH_I preparations from the batches NM 07 and NM 08 were combined to one preparation; in the text and figures this preparation is referred to as NM 0708_I.

Table 2 - II. Calculated efficiency of purification of human LH_I and LH_{II}.[1)]

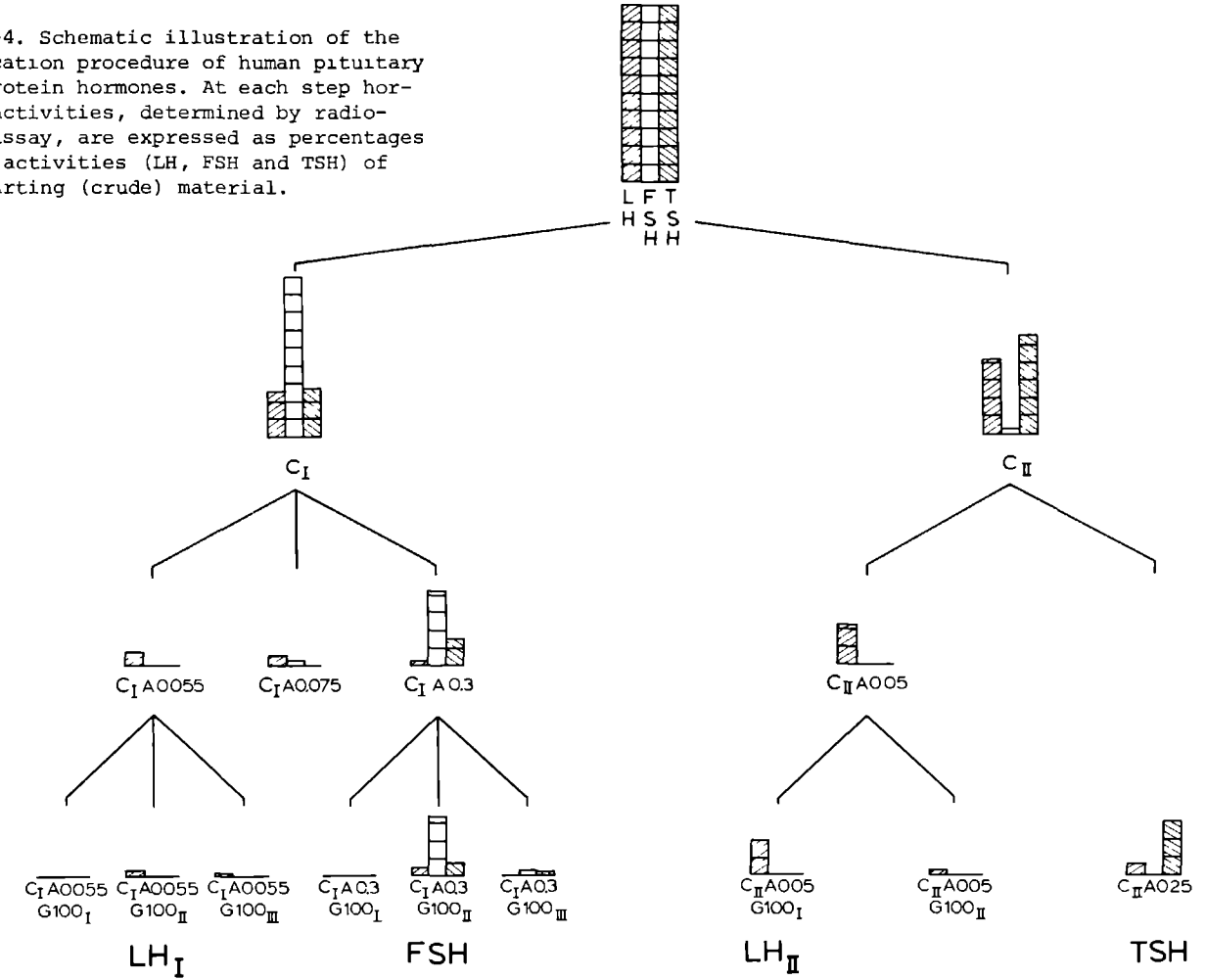
	Starting material	LH_I		LH_{II}	
Batch[2)]	Total initial biol. act. (IU 1st IRP LH bio)	Total final biol. act. (IU 1st IRP LH bio)	Final act / Initial act in %	Total final biol. act. (IU 1st IRP LH bio)	Final act / Initial act in %
NM 07	1.3×10^6	2.2×10^5	5.2	3.5×10^5	27.0
08	2.6×10^6			3.1×10^5	10.7
09	3.2×10^6	3.7×10^5	8.7	1.1×10^6	35.0
14	2.8×10^6	3.4×10^5	15.5	2.0×10^6	71.5
15	2.0×10^6	1.2×10^5	6.0	1.3×10^6	65.0
16	3.6×10^6	2.0×10^5	5.7	2.6×10^6	71.0
10	2.2×10^6	5.1×10^4	2.3	1.4×10^5	6.2
11	2.1×10^6	7.6×10^4	3.6	1.3×10^5	6.5

Comparable data:

Roos et al, 1975	25.0
Reichert & Parlow, 1964b	50.0
Rathnam & Saxena, 1970	75.0
Torjesen et al, 1975	19.0

1) For definition of terms, see text.
2) NM 07 - NM 16: acetone-dried glands;
NM 10 - NM 11: frozen glands.

Fig. 2-4. Schematic illustration of the purification procedure of human pituitary glycoprotein hormones. At each step hormonal activities, determined by radioimmunoassay, are expressed as percentages of the activities (LH, FSH and TSH) of the starting (crude) material.



To solve the question which purification method is quantitatively the most efficient, the activity of the purified preparations should be expressed as a percentage of the total activity of the starting material. Table 2 - II shows these percentages for biological activity. There is a striking distinction between the results of batches NM 10 and 11 (from frozen glands) and the others. The figures for the other batches are similar to those reported by *Reichert & Parlow (1964b), Rathnam & Saxena (1970), Jorgesen et al (1974)* and *Roos et al (1975)*.

Finally, to monitor the procedure as a whole, during the purification of several batches the immunological LH, FSH and TSH activities of the intermediate fractions were determined and expressed as percentage of the initial activities (Fig. 2 - 4). The data obtained confirm earlier conclusions that it is easier to obtain LH free from FSH than FSH free from LH. Also a substantial amount of TSH appears to remain in the FSH fraction. These data are all based upon specific RIA systems for the different hormones. However, inadvertent measurement of α/β-subunits cannot be completely excluded.

3.1. Introduction

The two forms of LH isolated differed with regard to their behaviour on ionexchange resins, from which it can be deduced that their isoelectric points differ. The isoelectric point (pI) of a protein has been defined as the pH at which the net electric charge of the total molecule is zero and the protein has zero mobility in an electrophoretic field. A glycoprotein consists of one or several chains of amino acids to which carbohydrates (pentoses and hexoses) have been attached. The net electric charge of a glycoprotein molecule is therefore the resultant of the individual charges of the amino acids and those of the carbohydrates. In LH the hexose whose contribution to the electrical charge is the largest, is sialic acid, which, moreover, is well-known to be essential for *in vivo* biological activity. A difference in pI between the two forms of LH may be caused by differences in amino acid composition, by differences in sialic acid content or by both. For a complete characterization of the LH preparations, determination of pI, sialic acid content and amino acid composition were deemed pertinent, particularly with a view to gain insight in structure-function relationships. It seemed of equal interest to determine whether there is a difference between LH_I and LH_{II} in molecular size. The mechanism of production and secretion of LH by the pituitary gland has been the subject of recent studies. *Hagen & McNeilly (1975)*,

Prentice & Ryan (1975), Edmonds, Molitch, Pierce & Odell (1975) and *Hagen, McNatty & McNeilly (1976)* have given evidence that a pool of α-subunits is available and that the β-subunits are produced when release is called for. Only through combination of the two subunits a biologically active LH molecule is formed. It may be expected that the pituitary glands used for the isolation of LH contained at least a certain amount of free α-subunits. To characterize the preparations as completely as possible the subunit contamination was, therefore, also determined.

3.2. Isoelectric point (pI)

Procedure. An LKB Instruments Inc. Multiphor polyacrylamide electrofocussing apparatus was used according to the conditions mentioned in the manufacturer's application note nr. 75. Ampholines were purchased from LKB, Bromma, Sweden. The components of the polyacrylamide gel for the pH range 3.5 - 9.5 consisted of 10 ml acrylamide solution 29.1% w/v, 10 ml NN'-methylene-bis-acrylamide solution 0.9% w/v, 36 ml sucrose solution 22% w/v, 2.8 ml Ampholine pH 4 - 6, 0.2 ml Ampholine pH 5 - 7 and 0.4 ml riboflavine solution 0.012% w/v. Polymerization was achieved under influence of UV light. The cathode solution was 1 M NaOH, the anode solution 1 M H_3PO_4. Electrofocussing occurred for 20 hours at 300 V. At the end of the run the pH gradient obtained was determined as described by *Finlayson & Chrambach (1971)*. This consists of freezing and slicing the gel, eluting the slices and measuring the pH of the eluate with a pH Radiometer Copenhagen. The gel slab was fixed and stained with a modified Coomassie Brillant

Blue solution, prepared according to *Malik & Berrie (1972)*. The LH_I and LH_{II} preparations of the batches NM 07 - 11 were investigated.

Results. In the pH range 3.5 - 10 the LH_I preparations showed a double band at pH 5.7 - 5.8; the LH_{II} preparations showed three bands, one major band at pH 8.8, the other two at pH 7.9 and 8.4 respectively (Table 3 - I). The experiments were repeated in other pH-ranges to check for development of artefacts due to possible interactions between the ampholines and the sample proteins *(Reichert, 1971)* but the results were essentially the same as before.

Table 3 - I. Isoelectric points (pI) and sialic acid content of purified human LH_I and LH_{II}.

	LH_I		LH_{II}	
Batch[1]	pI	% sialic acid (w/w)[2]	pI	% sialic acid (w/w)[2]
NM 07	5.7 - 5.8	3.2	7.9 - 8.4 - 8.8	2.1
08			7.9 - 8.4 - 8.8	2.3
09	5.7 - 5.8	3.1	7.9 - 8.4 - 8.8	2.8
14	n.d.[3]	3.6	n.d.[3]	3.0
10	5.7 - 5.8	1.2	7.9 - 8.4 - 8.8	1.4
11	5.7 - 5.8	1.5	7.9 - 8.4 - 8.8	1.3

1) NM 07 - NM 14: acetone-dried glands; NM 10 - NM 11: frozen glands.
2) Precision of the method, expressed as coefficient of variation: 3-5%.
3) n.d. = not determined.

3.3 Sialic acid

Procedure. Sialic acid was determined by the thiobarbituric acid method developed by *Warner (1959)*. In short, this method consists of treating the sample with hot diluted sulphuric acid to liberate the sialic acid, oxidizing it by periodate and coupling by thiobarbituric acid. The resulting chrorophore is then extracted in cyclohexanone and determined quantitatively by spectrometry at 549 nm. The LH_I and LH_{II} preparations of the patches NM 07 - 14 were investigated.

Results.[1] (Table 3 - I) It appears that in the case of acetone-dried material (NM 07, 08, 09 and 14) the sialic acid content was higher in the LH_I preparations than in the LH_{II} preparations, while that of the two LH preparations derived from frozen material (NM 10, 11) was more or less equal.

3.4. Amino acid analysis

Procedure. For practical reasons only one preparation of each of the four kinds (LH_I and LH_{II}, prepared from actone-dried glands; LH_I and LH_{II}, prepared from frozen glands) was analysed. In addition, the amino acid composition of the α- and β-subunits of LH_I and LH_{II} from acetone-dried glands were determined. The following preparations were chosen. NM 14_I, NM 01_{II}, NM 11_I and NM 11_{II}, as well as the subunits of NM 14_I and NM 01_{II}. After in vacuum hydrolysis of the proteins in 6 N HCl, amino acid compositions were determined according to *Spackman, Stein & Moore (1958)* using

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1) The sialic acid determinations were kindly carried out by Ir.J.A.Bakker

a Beckman amino acid analyzer model 121.

Results. (Table 3 - II) The data for NM 01_{II} and its subunits have been published earlier *(Closset, Van Dalem, Hennen & Lequin, 1975)*. The preliminary results of the other preparations indicate that the LH_I preparations contain more aspartic acid and glutamic acid but less proline and arginine than the corresponding LH_{II} preparations. Inspection of the figures for the subunit preparations suggests that the above differences are to be attributed to differences in the compositions of the α-subunits more than to those of the β-subunits.

Table 3 - II. Amino acid analysis[1] of purified human LH_I and LH_{II} and their subunits.

(Res/100 res)

Batch[2]	Arg	Asp	Glu	Pro
NM 14_I	4.7	9.5	10.9	7.7
NM 01_{II}	6.3	7.0	8.8	13.4
NM $14_I\alpha$	3.4	9.5	11.6	4.9
NM $01_{II}\alpha$	5.8	5.5	10.4	7.8
NM $14_I\beta$	9.5	6.5	8.3	15.0
NM $01_{II}\beta$	8.9	6.2	7.1	15.1
NM 11_I	4.2	9.1	11.2	7.3
NM 11_{II}	4.3	8.4	9.2	7.5

1) Only values, showing significant differences between intact LH_I and LH_{II} are presented.

2) NM 14 - NM 01: acetone-dried glands; NM 11: frozen glands.

3.5. Estimation of molecular size

It has been shown that during gelfiltration of proteins the elution volume, i.e. the amount of effluent measured from the application of the sample to the elution of the substance in maximal concentration, is approximately a linear function of the logarithm of the molecular weight *(Andrews, 1964, 1965)*. *Laurent & Killander (1964)* developed a mathematical formula to obtain the value for the partition coefficient (K_{av}), as defined by *Andrews (1964)* as follows: $K_{av}=(V_e-V_o)/(V_t-V_o)$, where V_e= elution volume of the protein, V_o= void volume of the column, V_t= total volume of the column. By plotting the logarithm of the molecular weight of known standard proteins against the corresponding value for K_{av} a standard curve is obtained. From this curve and the value for K_{av} for an unknown protein its approximate molecular weight can be derived.

Procedure. The final step in the purification of the LH preparations was gelfiltration on Sephadex G 100 (chapter 2). The columns had been calibrated beforehand by determining the void volume and total volume. The void colume was taken as the elution volume of a 0.1% Blue dextran solution, reading absorbancy at 540 nm, the total volume as the elution volume of a 0.5 M NaCl solution, reading conductivity with a conductivity Radiometer, Copenhagen. The elution volume of the LH preparations was derived from the elution profile as monitored both by protein determination and radioimmunoassay (Fig. 2-3).

Results. The values for K_{av}, although subject to some variation,

are higher for LH_I preparations (mean ± SE: 0.40 ± 0.01) than for LH_{II} preparations (mean ± SE: 0.25 ± 0.015). (Table 3 - III)

Table 3 - III. Partition coefficient (K_{av}) for column chromatography (Sephadex G 100) and subunit contamination for purified human LH_I and LH_{II}.

	LH_I			LH_{II}		
Batch[1)]	K_{av}	% α-subunit (w/w)	% β-subunit (w/w)	K_{av}	% α-subunit (w/w)	% β-subunit (w/w)
NM 07	0.40	14.5	9.0	0.18	40.7	10.2
08				0.26	39.8	11.6
09	0.41	8.0	6.0	0.19	32.8	8.9
14	0.41	12.1	7.2	0.25	18.2	1.5
15	0.40	39.0	16.0	0.30	8.4	3.3
16	0.42	20.4	17.5	0.28	4.7	2,3
10	0.34	12.0	9.0	0.27	7.6	4.1
11	0.41	17.0	10.0	0.27	2.5	1.8

1) NM 07 - NM 16: acetone-dried glands; NM 10 - NM 11: frozen glands.

From the standard curve presented by the manufacturer of Sephadex, quoted to be taken from *Andrews (1964)*, the molecular weights are estimated to be roughly 30000 for LH_I and 50000 for LH_{II}. According to *Andrews (1965)* the values for glycoproteins usually are overestimated due to the fact that glycoproteins do not have a strictly globular form; consequently, the precise molecular weights of the LH preparations remain to be determined.

3.6. Subunit contamination

Subunits can only be measured by radioimmunoassay, since they are not active in either the bioassay or the radioligand assay Direct measurement of the subunit content of an LH preparation is impossible because intact LH cross-reacts in systems employing antibodies against the individual subunits. It is imperative, therefore, to separate the subunits from the intact hormone. The difference in molecular weight makes a partial separation by G 100 gelfiltration possible. When the activity of the column fractions has been determined in the radioimmunoassay systems specific for LH_{α} and LH_{β}, the elution profile can be drawn. This is illustrated in Fig. 3 - 1 (lower part). Due to the above-mentioned differences in immunoactivity between intact LH and its subunits in their respective systems simple comparison of the areas under the peaks of intact hormone and subunit would lead to erroneous conclusions. This problem can be solved by comparing the elution profiles of an LH preparation with that of a highly purified subunit preparation. The area under the peak of the subunit preparation is put to 100%; the area under the second (= subunit-)peak of the intact preparation is then calculated relative to this figure.

Procedure. One µg of each LH_{I} and LH_{II} preparation and of highly purified LH_{α} and LH_{β} preparations, dissolved in 0.02 M PBS + 0.1% BSA, were applied to a Sephadex G 100 column (2 x 100 cm) equilibrated in this buffer. The immunological activity in the column fractions was determined by specific

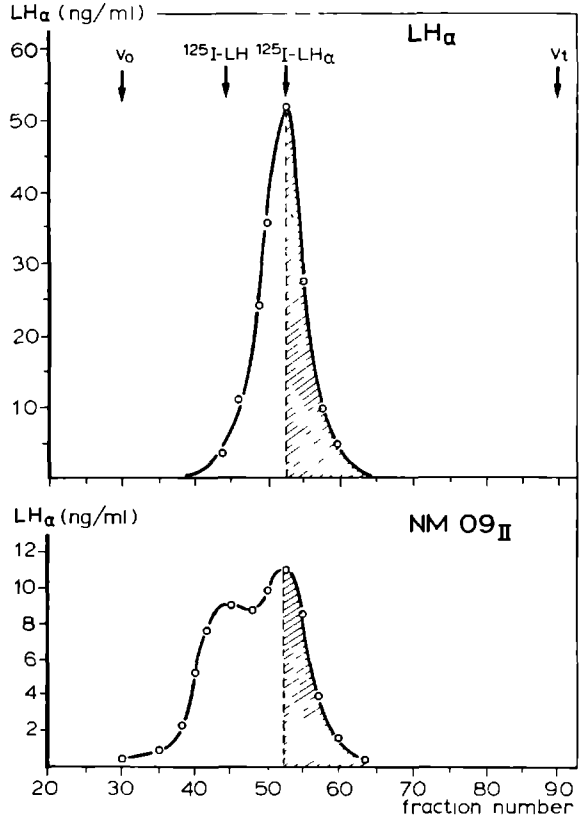


Fig. 3-1. Determination of subunit contamination by gelfiltration on G 100. As an example, the elution profiles of NM 09_{II} (lower part) and highly purified hLH_{α} (upper part), as assessed by a specific hLH_{α} radioimmunoassay system, are shown. Hatched areas were taken for calculation. See text for more details.

LH_{α} and LH_{β} radioimmunoassay systems (Table 7 - I). An example of the elution profiles thus obtained is shown in Fig. 3-1. Because the difference between the molecular weight of the intact hormone and that of the subunits is not sufficient for a complete separation, to calculate the area under the subunit peak the descending part was taken and multiplied by 2, assuming the peak to be symmetrical.

Results. The percentages for α- and ß-subunit contamination are presented in Table 3 - III. It is evident that the values for the α-subunit are higher than those for the ß-subunit, both in the LH_I and LH_{II} preparations. As it is well-known that the molecular weights of the subunits are approximately the same, our findings are in agreement with the concept that in the *in vivo* situation a pool of α-subunits is available, while the ß-subunits are produced when LH-release is called for *(Hagen & McNeilly, 1975; Prentice & Ryan, 1975; Edmonds et al, 1975; Hagen et al, 1976)*. The values obtained in this study, however, unlikely represent the *in vivo* condition of the pituitary gland, since a degree of dissociation post-mortem cannot be excluded. The results obtained here indicate that with regard to subunit contamination, apart from batch-to-batch variations, on the average no difference exists between LH_I and LH_{II}. Furthermore, when the figures concerning the acetone-extracted material (NM 07 - 09, NM 14 - 16) are compared with those concerning the frozen material (NM 10, 11) no significant difference appears to exist. Thus, the results do not point to an effect of the manner of preservation of the tissue.

3.7. Discussion

The results of divergent experiments were reported in this chapter. Of the various analyses, the pI determination and the estimation of the molecular size in particular indicate

that there are differences between LH_I and LH_{II}. Moreover, the results concerning the amino acid composition and sialic acid content are in direct agreement with those of the pI analysis: They all point to the fact, that LH_I is of a more acidic nature than LH_{II}. From the isoelectric focussing studies it also can be inferred that none of the preparations investigated was completely homogeneous. One could argue that the finding of several bands reflects the presence of intact hormone and free subunits. In that case the intensity of the presumed subunit bands would have varied with the degree of contamination. Although no quantitative analysis of the bands was made, such variation was at first glance not as obvious as one would expect on the basis of the large differences in subunit contamination (see Table 3 - III). Moreover, the LH_I preparations showed only two bands at pH values much lower than those for LH_{II}. From the amino acid analysis it could be concluded that the differences in composition between LH_I and LH_{II} mainly are to be attributed to differences in the amino acid composition of the respective α-subunits. As no appreciable differences in amino acid composition between the β-subunits were found, one would expect to find bands representing the β-subunits at approximately the same pH-values for both LH_I and LH_{II}. Since no such comparable bands could be detected, however, the appearance in the gel of subunits as separate bands is highly unlikely, at least for the β-subunits. It is more likely that the multiplicity of bands is caused by the presence of several molecular forms with a varying sialic acid content *(cf. Reichert, 1971)*. Coming now to the amino acid

compositions themselves, it should be realised that, although the observed differences are interesting, it is too early to draw conclusions. Firstly the preparations from acetone-dried glands were not from the same batch and, secondly, the figures for LH_I and LH_{II} might be influenced by (varying) amounts of contaminating subunits. Yet, the preparations from frozen glands which were from the same batch (NM 11) and, moreover, contained less subunits, showed also differences, albeit to a lower degree.

Further evidence that LH_I and LH_{II} are different entities stems from the gelfiltration studies. Since the reported K_{av}-values are those for the intact LH molecules, in this case disturbing influence of contaminating subunits is excluded. The difference in molecular size between LH_I and LH_{II} might have several causes e.g. the secretion of several molecular forms of LH by the pituitary gland during life, or it might be brought about by the splitting off of part of the molecule induced by either post-mortem proteolysis or by conditions of preservation of the glands. By determination of the primary structure of the protein chain of LH_I this and a number of other questions could be elucidated. Unfortunately, structure determination consumes a large amount of each preparation. Until now, a sufficient quantity of LH_I prepared from one single batch of pituitary glands was not available.

As mentioned in chapter 1, in this investigation four different assay methods were used to characterize the LH preparations, namely the *in vivo* bioassay, the *in vitro* bioassay, the radioimmunoassay (RIA) and the radioligand assay (RLA). In this chapter attention is paid to differences and resemblances between them with regard to particular requirements and procedure, as well as concerning analysis of their results (Table 4-I).

Table 4-I. Comparison of characteristics of four different hormone assay methods.

Methods / Characteristics	*In vivo* bioassay	*In vitro* bioassay	RIA	RLA
Standard hormone	+	+	+	+
Unknown preparation	+	+	+	+
Labelled hormone	-	-	+	+
Level of target	animal	organ/tissue	antibody molecule	cell membrane
Measurement of effect	indirect: by separate method	indirect: by separate method	direct	direct

The starting point for all four methods is the comparison of the responses elicited by a standard hormone preparation and those of an unknown preparation. Thus, the potency of the

unknown preparation is expressed in terms of the standard. In the *in vivo* and *in vitro* bioassay the response is a certain change within the test animal (*in vivo* bioassay) or within the isolated target organ (*in vitro* bioassay) and subsequently is measured by a separate appropriate method. In the RIA and RLA the response is based on the capability of both the standard and the unknown preparation to compete with a labelled hormone for binding sites. In the RIA these binding sites are on the antibodies, in the RLA, they are present on the membranes of the target organ cells. In either case the response is measured directly. For analysis of results the responses measured must be correlated in some way to the dose of hormone used. The ultimate mathematical expression of this dose-response relationship may vary with the assay method,but to simplify calculation of potencies of unknown preparations as well as estimation of the error of the method, one strives for a relationship, which is linear over at least part of the dose range. All hormone assays are based upon the assumption that the standard and the unknown preparations behave identically, i.e. as if they were dilutions of each other *(Bangham & Cotes, 1971, 1974)*. Therefore, after having established the regression lines for the standard and unknown preparations, the parallellism of these lines must be investigated statistically. Whereas parallellism of the regression lines is only an *indication* of identical behaviour, non-parallellism is *proof* of non-identical behaviour. From the distance between the lines, measured along the dose-axis the

potency of the unknown preparation relative to the standard, can be computed.

Apart from parallellism each method has to be tested for its reliability. In general, reliability consists of four components namely accuracy, precision, specificity and sensitivity *(Borth, 1957)*.

Accuracy is defined as the nearness with which a given analytical result approaches reality. It cannot be measured for assays of protein hormones, because the exact molecular hormone concentration is not known. This, in contrast to assays of e.g. steroid hormones where absolute concentrations can be determined by methods like gas-chromatography and mass-spectrometry.

Precision may be defined as the standard deviation from the mean value of a series of determinations. *Gaddum (1933)* introduced the index of precision, λ, which is defined as the standard deviation from the mean of all responses (s) devided by the slope of the regression line (b). The precision is high when s is small and b is large, i.e. when λ (=s/b) is small. To give an impression of the order of magnitude, by experience the value of λ for an *in vivo* bioassay should not exceed 0.3, for a RIA λ should not exceed 0.1. Another way to obtain an impression of the precision of an assay is to calculate the fiducial limits, i.e. the upper and lower limits of the range within which the calculated mean lies with a certain probability. The smaller the range, the higher the precision. Usually the fiducial limits are calculated for

the 95% probability level. The mathematics are not as simple as in the case of λ and may be obtained from standard textbooks e.g. *Ermens (1948), Finney (1964), McArthur & Colton (1970).*

Specificity is defined as the determination of one particular entity to the exclusion of others. For protein hormones this component is probably the most complex one. For details concerning specificity for each of the four methods used, the reader is referred to the appropriate chapter.

There exists disagreement as to how to define *sensitivity*. Some investigators define sensitivity as the slope of the standard curve, others as the minimal detectable dose. Sensitivity in either sense is an important concept wher samples which have a minimal hormone content must be measured. In the present investigation, however, the hormone concentrations in each preparation were comperatively high and, thus, the sensitivity of the assay methods used was of lesser concern.

5.1. Introduction

A hormone essentially is defined by its biological effects in the living organism. A bioassay is an assay based upon these effects with the purpose to assess the potency of any preparation of the hormone in comparison with a standard preparation of the same hormone. There are two types of bioassays: (1) those based on quantal effects whereby the endpoint concerns an all-or-nothing effect, e.g. survival or death, weight increase above a certain level etc.; (2) those based on graded effects whereby the effects can be quantified e.g. body weight, organ weight, number of cells produced etc. In this investigation only bioassays of the second type have been applied. When carrying out these assays known quantities of both the standard and the test preparations are administered, the effects are measured and dose-response curves are constructed.

5.2. Survey of the most common *in vivo* bioassays for LH and FSH

5.2.1. Bioassays specific for LH

Greep, Van Dyke & Chow (1941) reported a method based upon the LH-induced enlargement of the ventral prostate in hypophysectomised, immature male rats (VPW-test). *Watts & Adair (1946)*

used the LH-induced weight increase of the seminal vesicles in hypophysectomised, immature male rats (SVW-test). Although this assay is less sensitive than the VPW-test, it has the advantage that it can also be carried out with intact immature rats *(Diczfalusy & Lorraine, 1955; Van Hell, Matthijsen & Overbeek, 1964)*. *Diczfalusy (1954)* introduced a test based upon the fact that LH causes a weight increase of the total accessory reproductive organs in intact immature rats (WITARO-test). *Parlow (1961)* used the ovarian ascorbic acid depletion (OAAD) by LH in immature pseudo-pregnant female rats as an end-point. In this assay pseudo-pregnancy is induced by pre-treatment with PMS and hCG.

There is a distinction between the VPW-, SVW- and WITARO-tests on the one hand and the OAAD-test on the other: the latter is an acute assay in which the effect of a particular dose of hormone is measured after a limited period of time (a few hours); the former are chronic assays in which the hormone is administered in several dosages over an extended period of time (days) and the effect is measured still later. As a consequence, hormone preparations which are metabolized quickly often have an apparent higher potency in the acute assay than in the chronic assay.

5.2.2. Bioassays specific for FSH

Steelman & Pohley (1953) developed a method upon the ovarian weight increase induced by FSH and augmented by hCG in intact immature rats (AOW-test). *Brown (1955)* modified this assay

for application in mice.

5.2.3. Assays for total gonadotropic activity

Klinefelter, Albright & Griswald (1943) first described a method to test total human urinary gonadotropins possessing both LH and FSH activity whereby the uterine weight increase of intact immature mice was measured. This test is now known as the Mouse uterus test for GSH (=gonad stimulating hormone) to indicate that neither LH nor FSH is measured specifically.

5.3. Bioassays used in this investigation

For LH the OAAD-method *(Parlow, 1961)* was used; some preparations were also assayed with the SVW-test *(Van Hell et al, 1964)*. The OAAD-method was carried out as follows. Immature female Wistar rats, 27 days old and weighing 40 - 50 g, were obtained from the Central Institute for the Breeding of Laboratory Animals (TNO, Zeist, the Netherlands). They were pretreated with PMS (Gestyl®, Organon) injected subcutaneously (s.c.) in a dose of 50 IU in 0.2 ml distilled water + 0.1% BSA (Sigma), followed 65 hours later by hCG (Pregnyl®, Organon) 25 IU in 0.2 ml distilled water + 0.1% BSA (Sigma). The purpose of the pretreatment with these hormones is to induce pseudo-pregnancy and to increase and maintain the ovarian ascorbic acid content. The significance of this increase is to enhance the sensitivity of the assay: the higher the ascorbic acid level, the smaller the amount of LH that causes a measurable ascorbic acid depletion. In a pilot experiment it could be shown that the increased ascorbic acid

level was maintained from the 4th to the 9th day after the hCG injection. Therefore, on the morning of either the 5th or 6th or 7th day after hCG injection the animals were injected intravenously (i.v.) with standard LH or test material dissolved in saline + 1% BSA (Sigma). Exactly 4 hours after this injection each rat was killed after ether anaesthesia, the right-hand ovary was carefully dissected out, weighed and homogenized in 10 ml icecold 2.5% metaphosphoric acid (MPA). The homogenate was filtered twice through Whatman paper no 41. The filtrate was kept frozen. The ascorbic acid concentration was determined by a modification of the method of *Windlin & Butler (1968)*, using as reagents 2,6-dichloroindophenol (DCIP; 49.2 mg/l) and Na-acetate (45.3 g/l), adjusted to pH 7.0. Equal volumes of the two reagents were mixed immediately before use and kept cold. Five ml of this mixture was added to 3 ml of the filtrate and mixed during 30 s. The absorbancy at 515 nm was read on a Coleman Junior spectrophotometer with MPA as a blank. A calibration curve of ascorbic acid was made as follows: a stock solution of ascorbic acid in MPA 0.1 mg/ml was diluted to contain 3, 6, 12 and 24 µg/ml resp. Three ml of each of these solutions were mixed with the DCIP-acetate reagent and the absorbancy measured was plotted versus the ascorbic acid concentration. The ascorbic acid content of each filtrate was computed and expressed per unit ovarian weight. Dose-response curves were constructed by plotting the log dose versus the ascorbic acid content in µg per 100 mg ovarian weight.

The SVW-method was carried out as follows: immature male Wistar rats, approx. 25 days old and weighing 35 - 45 g, were obtained from the Central Laboratory for the Breeding of Laboratory Animals (TNO, Zeist, the Netherlands). They were injected s.c. with standard or test material dissolved in saline + 1% BSA (Sigma) once per day always at the same time on three

consecutive days. On the fourth day the animals were killed, the seminal vesicles were carefully dissected out and weighed. Dose-response curves were constructed by plotting the log dose versus the seminal vesicle weight.

For FSH the ovarian augmentation test (AOW-test) as described by *Steelman & Pohley (1953)* was used. Immature female Wistar rats, approx. 25 days old and weighing 30 - 40 g, were obtained from the Central Laboratory for the Breeding of Laboratory Animals (TNO, Zeist, the Netherlands). They were injected s.c. 6 times on three consecutive days (once on the first day: at 3 p.m.; three times on the second day: at 9 a.m., noon and 3 p.m. and twice on the third day: at 11 a.m. and 3 p.m.) with 3 doses of standard or test material dissolved in 0.02 M phophate buffer pH 7.1 + 1% BSA (Sigma). This last buffer also contained hCG (Pregnyl®, Organon) in such a concentration that each animal received a total of 60 IU (established in pilot experiment). Litter-mates were used whenever possible. The injection schedule was such that one animal of each pair of litter-mates received a dose of the standard preparation and the other one a corresponding dose of the test solution. Since a pilot study had shown that the slope of the dose-response curve was maximal when the ovarian weights were determined approximately 45 hours after the last injection, in all experiments the rats were killed on the fifth day after the first injection and both ovaria were carefully dissected out and weighed. Dose-response curves were constructed by plotting the log dose versus the ovarian weight.

Calculation of biological activity; reference preparations

In each assay a local standard was used after calibration against the

Table 5 - I. Potencies of human LH_I and LH_{II} determined by *in vivo* bioassay (OAAD)[1]

Batch[2]	LH_I IU 1st IRP LH/FSH bio/mg (mean; 95% fiducial limits)			LH_{II} IU 1st IRP LH/FSH bio/mg (mean; 95% fiducial limits)		λ
NM 07				8590 (1500-37820)		0.33
	11580 (6370-35190)	13650 (10330-25670)	0.28	3990 (1490- 8200)	6040 (4610- 8250)	0.40
	15690 (10510-26810)		0.24	6140 (4420- 8890)		0.18
08				1690 (280- 3390)		0.35
				1380 (130- 2980)	1490 (130- 3120)	0.43
				2470 (910- 6300)		0.40
09	7010 (5200-10440)	7860 (5390-11370)	0.17	4280 (2930- 6460)	4390 (3050- 6560)	0.21
	8060 (5640-12840)		0.20	4520 (3200- 6570)		0.21
14	3570 (2300- 5800)	4690 (3320- 6930)	0.24	9250 (6070-17750)	8400 (6630-11100)	0.22
	5760 (3320-12620)		0.35	8370 (6200-11730)		0.17
15	9420 (6920-13280)	9420 (6920-13280)	0.18	12560 (8170-24230)	12560 (8170-24230)	0.23
16	3070 (1800- 4530)	3070 (1800- 4530)	0.23	9550 (6310-16270)	9550 (6310-16270)	0.24
10	5130 (2410- 6890)	5130 (2410- 6890)	0.19	4580 (1180- 9270)	4580 (1180- 9270)	0.41
11	2940 (1380- 4890)	2940 (1380- 4890)	0.23	3230 (23-12510)	3700 (1620- 6040)	0.40
				3740 (1650- 6030)		0.24
	Combination 07-16:	7400 (6370- 8710)		Combination 07-16:	5650 (4940- 6480)	
	10-11:	4280 (2830- 6150)		10-11:	3830 (1480- 6920)	

1) OAAD: Ovarian ascorbic acid depletion test
2) NM 07 - NM 16: acetone-dried glands; NM 10 - NM 11: frozen glands

ınternatıonally accepted standard. The 26th WHO expert committee has establıshed the preparatıon known as MRC 69/104 as the 1st IRP for use ın bıoassay both for LH and FSH, wıth an assıgned potency of 25 IU LH per ampoule and 10 IU FSH per ampoule *(WHO ECBS 26th report, 1975)*. As local standard for LH the preparatıon NM 07 type II was used and for FSH a batch of crude human pıtuıtary materıal coded HHS. The results of the bıoassays were statıstically evaluated usıng a computer programme based on the "parallel lıne" method descrıbed by *Brownlee (1960)*. The results of repeated tests of a particular assay were combıned usıng a computer programme based on a method by *Finney (1964)*. Both computer programmes were kındly made avaılable by Dr. W. Brıdson (Natıonal Institute of Health, Bethesda, U.S.A.).

5.4. Results and dıscussıon

FSH. When assayed ın the AOW-test all LH preparatıons had an FSH actıvıty of less than 10 IU 1st IRP LH/FSH bıo/mg.
LH. Table 5 - I shows the results of the OAAD-tests. The values for the ındex of precısıon, λ, varıed between 0.2 and 0.4, whıch corresponds to the fıgures reported ın the lıterature *(Koed & Hamburger, 1968; Schmıdt-Elmendorff & Lorraıne, 1962)*. The results for the LH_I and LH_{II} preparatıons show a consıderable batch to batch varıatıon. For some batches LH_I has a greater potency, for others LH_{II} ıs more potent. When the results of all batches from acetone-drıed glands (NM 07, 08, 09, 14, 15 and 16) are combıned, the LH_I preparations appear to be more potent than the LH_{II}

Table 5 - II. Potencies of human LH_I and LH_{II} determined by *in vivo* bioassay (SVW)[1]

Batch[2]	LH_I IU 1st IRP LH/FSH bio/mg (mean; 95% fiducial limits)	λ	I.D. (OAAD/SVW)	LH_{II} IU 1st IRP LH/FSH bio/mg (mean; 95% fiducial limits)	λ	I.D. (OAAD/SVW)
NM 07	12900 (7790-18630)	0.17	1.1	3330 (2450-5120)	0.20	1.8
08				690 (400-1120)	0.27	2.2
09	5710 (3180-11620)	0.30	1.4	3110 (2050-5100)	0.23	1.4
14	3860 (2500- 6050)	0.24	1.2	2360 (1560-3210)	0.17	3.5
15	3700 (1560- 5790)	0.21	2.5	1090 (270-2000)	0.25	11.5
16	2690 (1760-33790)	0.41	1.1	2570 (1090-3980)	0.18	3.7
10	4520 (2980- 7790)	0.20	1.1	2070 (1060-3570)	0.31	2.2
11	1490 (910- 2780)	0.32	2.0	1770 (760-3010)	0.80	4.6
Combination						
07 - 16:	5660 (3220-11040)			2210 (1280-4120)		
10 - 11:	2580 (1340- 5810)			1920 (630-4560)		

1) SVW: Seminal vesicle weight test
2) NM 07 - NM 16: acetone-dried glands; NM 10 - NM 11: frozen glands

preparations, but there is some overlap of the 95% fiducial limits. Similarly, combination of the results of the batches NM 10 and 11 (from frozen glands) show that in these preparations there is no statistically significant difference between the potencies of LH_I and LH_{II}.

Since the introduction of the OAAD-test by Parlow there have appeared many reports pro and contra this method, in particular by those who question the specificity. Indeed, it has been shown that other substances also could produce an ascorbic acid depleting effect e.g. vasopressin *(McCann & Taleisnik, 1960)* and serum from hypophysectomised rats *(De Groot, 1967)*. Although these effects certainly must be taken into account when the measurement of LH in body fluids is considered and even constitute an impediment for the use of the OAAD-test for this purpose, it does not mean that the OAAD-test is unfit to measure LH in highly purified preparations.

It should be realised that, as pointed out before, the OAAD-test is an acute assay. It may, therefore, be measuring a hormone component different from a component active in a chronic assay. For this reason it seemed of interest to assay the LH preparations also in a chronic test. The SVW-test was chosen, since it is much easier to perform than the classical VPW-test and *Diczfalusy & Lorraine (1955)* had shown that the two tests compare favourably. The results are compiled in Table 5 - II. For some unknown reason, the slopes of the dose-response curves were very flat, resulting in rather high

values of λ as compared to those reported in literature. This discrepancy may be related to the strain of animals used. These results of the SVW-test confirm the differences in potencies of LH_I and LH_{II} preparations, as revealed by the OAAD-test. The two methods may be compared by calculating for each preparation the index of discrimination (I.D.) i.e. the ratio of the potencies found in OAAD and SVW. The I.D.-values are presented in Table 5 - II. For the LH_{II} preparations these values appear to be higher than for the LH_I preparations. This phenomenon could be explained as follows. The sialic acid content of the LH_{II} preparations was found to be lower than that of the LH_I preparations (Table 3 - I). In the glycoproteins, sialic acid shields the galactose residue, which forms the point of attack for catabolism by the liver. As was shown for hCG *(Van Hall, Vaitukaitis, Ross, Hickman & Ashwell, 1971)* removal of the sialic acid shortens the metabolic half-life; consequently, the potency is decreased more in chronic assays than in acute assays. Though applicable for the preparations from acetone-dried glands, this reasoning does not hold quite for the preparations from frozen glands (NM 10-11) because here no differences in sialic acid could be shown to exist between the LH_I and LH_{II} preparations.

6.1. Introduction

The major drawback of *in vivo* bioassays is their relative insensitivity. During the last decade there has been a search for more sensitive bioassays, using as a starting point the physiological chain of events initiated when the hormone reaches its target organ. This chain usually ends with the production of one or several compounds, in the case of LH e.g. steroids. An *in vitro* bioassay is an assay based on the production of such compounds by the isolated target organ after incubation with the effective hormone. Instead of the whole organ, also tissue slices or cell suspensions can be used. It is self-evident that a proper *in vitro* bioassay can be performed only if the production of the compound is quantitatively related to the graded doses of the hormone. Advantages of *in vitro* bioassays as compared to *in vivo* bioassays are: an increase in sensitivity combined with a reduced variability and the absence of possible disturbing metabolic effects. In addition to its usefulness for quantitative determinations, target organ material can also be used to elucidate the mechanism of action of the hormone. From numerous investigations it may be concluded that the above-mentioned chain of events for most hormones is as follows: the hormone binds to certain specific sites on the membrane of the target cells; the membrane-bound enzyme adenylcyclase is activated and catalyses the formation of cyclic AMP (cAMP) from ATP; cAMP

activates protein kinases which, in turn, activate phosphorylating enzymes, which, then, catalyse the formation of a final product. Since cAMP in most cases mimicks the action of the protein hormone it is called "the second messenger". For recent reviews see *Sutherland, 1971; Major & Kilpatrick, 1972; Marsh, 1975.*

For the gonadotropins LH and FSH the obvious target organs are the gonads. For *in vitro* bioassay the use of material from the ovary as well as from the testis has been reported. Usually, these bioassays are based on the production of steroids but also other activities may be determined, e.g. lactate production, amino acid uptake and uridine incorporation *(Armstrong, 1968; Jarlstedt, Nilsson, Hamberger & Ahren, 1973; Herlitz, Hamberger & Ahren, 1976; Nilsson & Selstam, 1976)*. Although FSH is required for physiological events such as follicle growth or spermatogenesis, it is difficult to quantify these processes. Hence, the development of an *in vitro* bioassay for FSH is hampered. LH has been shown to regulate steroidogenesis, e.g. testosterone production by the Leydig cells and progesterone production by the luteinized granulosa cells. Assay systems for LH based upon the dose-dependent steroidogenesis have been successfully employed *(Watson, 1971; Dufau, Catt & Tsuruhara, 1972; Van Damme, Robertson & Diczfalusy, 1974)*. Since LH also increases the concentration of cAMP in a dose-dependent fashion, it is possible to take cAMP determination as an alternative end-point for the *in vitro* bioassay of LH (see review by

Marsh, 1975). Thus, the bioassay of LH consists of incubating a series of samples of the target tissue with increasing doses of LH and subsequent determination of the steroid or cAMP produced by means of radioimmunoassay or the technique of competitive protein binding. By plotting the amount of product versus the log dose of LH a standard curve is obtained. Concerning the reliability of this type of assay the same criteria apply as outlined in chapter 4. The hormone specificity is assured as long as steroid production is taken as an end-point. However, in the case of cAMP determination it must be kept in mind that this compound is involved in nearly all protein-hormone induced processes. Therefore, cAMP determination may be used provided the hormone preparation is highly purified. Alternatively, if contamination with other hormones is suspected, a highly specific target tissue must be employed.

6.2. *In vitro* bioassays used in this investigation

6.2.1. Progesterone and cAMP in porcine granulosa cells

Minimal intra-assay variation requires homogeneity of the target tissue. Cell suspensions are, therefore, to be preferred over tissue slices. However, dispersion of cells from testis or luteal tissues - structures commonly used to assay LH - necessitates firm treatment with enzymes like trypsin or collagenase which may well damage the cells. Since

C.P. Channing and her collaborators had shown that granulosa cell suspensions respond to LH with the production of cAMP and progesterone (see review by *Channing & Tsafriri, 1977)*, it was decided to develop an *in vitro* bioassay for LH, using such granulosa cells. Suspensions of granulosa cells are readily prepared without enzyme treatment. Pig ovaries were used as a source as these organs are easily available from the slaughterhouse.

6.2.1.1. Preparation of cell suspensions

Suspension of porcine granulosa cells was carried out as described by *Channing & Ledwitz-Rigby (1976)*. Ovaries from immature pigs, 4 - 6 months old, were collected at the slaughterhouse within one hour after killing of the animals, and kept on ice during transfer to the laboratory. Cells from medium-sized follicles (3 - 5 mm) were harvested by aspiration with a 20 - 23 gauge, 1 inch needle connected to a water pump applying very gentle suction. The cells were separated from the follicular fluid by centrifugation, 5 min. at 600 g. The pellet was resuspended in Krebs Ringer bicarbonate buffer pH 7.4, containing 0.2% glucose and 0.1% BSA (KRBGA buffer). The cells were washed twice in this buffer and resuspended in a volume of 2 ml. After counting the number of cells in an aliquot of this suspension using a haemocytometer, the final volume of the suspension was adjusted to a concentration of 1 x 10^6 cells per 50 µl.

6.2.1.2. Assay procedure

The incubation mixture, pipetted in disposable round-bottommed glass tubes

(6 x 50 mm) consisted of 50 µl of cell suspension and 450 µl of KRBGA buffer containing 1 - 1000 ng of an LH preparation (standard or unknown). A few incubation mixtures without LH served as controls. Incubation was carried out at 37°C under a 95% O_2 + 5% CO_2 atmosphere during 1 h if cAMP was to be measured, or during 20 h if progesterone was taken as an end-point. At the end of the incubation period the tubes were centrifuged and the cell pellet (for cAMP determination) or the supernatant (for progesterone measurement) was saved.

6.2.1.3. Determination or cAMP and progesterone

To prevent enzymatic degradation of the *cAMP* by phosphodiesterase, the cell pellet was resuspended in 500 µl of 50 mM Na-acetate pH 4.0 and the tubes placed in boiling water for 3 minutes. The cells were homogenized by hand. The homogenate was centrifuged, 5 min. at 2000 g, and an aliquot of the supernatant was used for the cAMP assay *(cf. Lamprecht, Zor, Tsafriri & Lindner, 1973)*. The cAMP was measured by a competitive protein binding assay using the method of *Gilman (1970)* that was modified at three points: (1) bovine kidney instead of bovine muscle was used as source for the binding protein; (2) in the isolation procedure of the binding protein chromatography on DEAE-cellulose was omitted; (3) no protein kinase inhibitor was used.
Progesterone was measured by a specific radioimmunoassay, described by *Thomas, Corbey & Rolland (1977)*, in an n-hexane extract of the incubation medium.

6.2.1.4. Results and discussion

In the preliminary investigations an LH_{II} preparation (NM 14)

was used. Although in all experiments LH caused a rise in progesterone levels in the medium it appeared to be impossible to obtain a dose-response correlation. (Recent experiments by *Channing (personal communication)*, similarly, revealed that LH promoted progesterone secretion but that the effect was not dose-dependent). The same experiments, however, revealed that LH invoked an increase in the level of cAMP in a dose-dependent way. It is difficult to explain these findings. *Channing (1970)* has shown that porcine granulosa cells from medium-sized follicles do not spontaneously luteinize in culture, but only after stimulation by LH. Our finding of cAMP stimulation one hour after adding LH is in agreement with the current concept that cAMP is indispensable for the initiation of the luteinization proces *(Channing & Seymour, 1970; Kolena & Channing, 1972; Channing, 1974)*. Since under the conditions of this experiment it takes at least 12 - 16 hours for complete luteinization of granulosa cells, it must be assumed that an equal period of time must elapse before progesterone secretion attains its maximum. Analysis of the progesterone production in time suggests that the cells are still active 10 - 12 hours after commencing incubation. However, the possibility of death or "dysfunction" of part of the cells cannot be excluded.

The foregoing experiments were carried out during the summer months. Unfortunately, continuation of the experiments from the end of August till October yielded completely negative results: neither progesterone nor cAMP production

could be enhanced by LH. Whether this must be attributed to seasonal variation (concerning temperature, light, food, treatment of the animals etc.) of to some other factor could not be established.

This unsatisfactory situation prompted us to investigate whether a modification of the same assay would yield better results.

6.2.2. Progesterone and cAMP in *intact* porcine follicles

Studies reported by Lindner's group show that incubated intact rat Graafian follicles respond to LH with cAMP and progesterone production *(Lindner, Tsafriri, Lieberman, Zor, Koch, Bauminger & Barnea, 1974)*. The advantage of dissecting the whole follicle is that the follicular structure remains intact and that individual cells are not damaged. The disadvantage, however, is that the population is less homogeneous than a cell suspension. In spite of this last objection, which counts heavily in a quantitative determination, the feasibility of using intact porcine follicles of medium size was investigated.

6.2.2.1. Tissue preparation

Ovaries from immature pigs were collected as described in section 6.2.1.1. Intact, medium-sized follicles (3 - 5 mm) were carefully dissected out. They were rinsed twice in Krebs Ringer bicarbonate buffer pH 7.4, containing 0.2% glucose and 0.1% BSA (KRBGA buffer).

6.2.2.2. Assay procedure

The incubation mixture, pipetted in disposable round-bottommed glass tubes (6 x 50 mm) consisted of 500 µl KRBGA buffer containing 50 - 500 ng of an LH preparation (standard or unknown). A few incubation mixtures without LH served as controls. The follicles were divided at random over the tubes, one follicle per tube. On the basis of the results of a series of pilot studies, in which an LH_{II} preparation (NM 14) was used, incubation was carried out at 37°C under a 95% O_2 + 5% CO_2 atmosphere during 1 h if cAMP was to be measured, or during 4 h if progesterone was taken as an end-point. At the end of the incubation period the medium was removed by aspiration and the follicles were saved for cAMP or progesterone determination.

6.2.2.3. Determination of cAMP and progesterone

The follicles serving *cAMP* determination were treated as follows. To prevent enzymatic degradation of cAMP by phosphodiesterase, 500 µl 50 mM Na-acetate pH 4.0 was added to the tubes containing the follicles. These tubes were then immersed in boiling water for 3 minutes. Fragmentation of the follicles was ensured by squeezing and scraping with a glass rod. After centrifugation (5 min. at 2000 g) a portion of the supernatant was used for the cAMP assay as described in section 6.2.1.3.
To the follicles to be used for *progesterone* measurement, 500 µl of distilled water was added. The follicles were then fragmented as described above. The contents of each tube were extracted with n-hexane and the progesterone determination carried out according to *Thomas et al (1977)*.

6.2.2.4. Results and discussion

It may be unusual to measure progesterone in the follicular fluid rather than in the incubation medium. However, comparative pilot studies showed that the LH-induced rise in progesterone secretion was the same in these two compartments. As the radioimmunoassays of progesterone seemed to be less subject to aspecific effects in the case of the follicular fluid, analysis of this last compartment was preferred. Another important result is that the incubation time optimal for progesterone determination appeared to be 4 h, which is much shorter than when granulosa cell suspensions were used (20 h). The faster response to LH by intact follicles, as compared to that by granulosa cell suspensions, is probably related to the presence of the theca cells. Theca cells of immature follicles, unlike granulosa cells, are capable of steroidogenesis *(Guraya, 1971; Younglai, 1972; Weiss, Seamark, McIntosh & Moor, 1976)*. They are, moreover, in direct contact with the LH-containing medium. The important role of the theca was confirmed in an experiment whereby the response of intact follicles was compared with that of punctured follicles from which the granulosa cells had been removed by scraping the follicular wall. (For details on this latter technique see *Channing & Ledwitz-Rigby, 1975*.) There was hardly a difference between the progesterone production in the two groups.

Unfortunately, the over-all results indicated that the progesterone production by intact follicles was not quanti-

Table 6 - I. Potencies of human LH_I and LH_{II} determined by *in vitro* bioassay. (cAMP production in porcine follicles)

Batch[1]	LH_I IU 1st IRP LH/FSH bio/mg (mean; 95% fiducial limits)		λ	LH_{II} IU 1st IRP LH/FSH bio/mg (mean; 95% fiducial limits)		λ
NM 07	3830 (1340-11050)	4260 (2200-8440)	0.57	2530 (1340- 4410)	2590 (1390- 4340)	0.27
	4950 (2380-11240)		0.39	3110 (420- 8860)		0.56
08				3610 (1070-10240)	3610 (1070-10240)	0.41
09	4110 (1420-12510)	3730 (1300-7650)	0.58	4720 (500-55480)	4440 (1740-12280)	0.86
	3070 (1690- 5330)		0.31	4220 (2300- 8170)		0.33
14	2340 (1500- 3450)	2460 (1690-3490)	0.19	5740 (2380-24450)		0.50
	3110 (1190- 7480)		0.46	2560 (1060- 6000)	3840 (2430- 6300)	0.44
				3960 (2020- 8490)		0.36
15	3110 (920- 7060)	2750 (820-5450)	0.41	5990 (3380-11740)	4300 (3100- 7610)	0.29
	2260 (1480- 3220)		0.42	3030 (1730- 5100)		0.28
16	3340 (1150- 9090)	1340 (460-2690)	0.47	2070 (730- 4370)	2510 (1170- 4540)	0.43
	650 (40- 1690)		0.58	3410 (720- 8830)		0.30
10	2420 (1270- 4180)	2070 (1210-3270)	0.28	2650 (1610- 4450)	3570 (2500- 5280)	0.24
	1230 (230- 2950)		0.53	4870 (2920- 9360)		0.25
11	3720 (1300-13030)	2010 (1020-3690)	0.48	2300 (1270- 4340)	2880 (1830- 4730)	0.29
	1270 (460- 2530)		0.40	3680 (1840- 8940)		0.34
	Combination 07-16: 2620 (1920-3490)			Combination 07-16: 4190 (3060- 5680)		
	10-11: 2060 (1780-2640)			10-11: 3610 (2720- 4880)		

1) NM 07 - NM 16: acetone-dried glands; NM 10 - NM 11: frozen glands

tatively related to the dose of LH. The progesterone determination was, therefore, unfit as an end-point for the *in vitro* bioassay of LH and consequently it was rejected. On the other hand, for the LH effect on cAMP a dose-response relationship was shown to exist. It has been reported that not only LH but also FSH stimulates cAMP production by intact rat follicles *(Lindner et al, 1974; Lieberman, Barnea, Bauminger, Tsafriri, Collins & Lindner, 1975)*. In view of the very low FSH content of our LH preparations, as was established by *in vivo* bioassay *(chapter 5)*, any FSH-induced cAMP production was considered insignificant. As local standard NM 14_{II} was chosen. This preparation, calibrated against the 1^{st} IRP LH/FSH bio (formerly known as MRC 69/104), had a potency of 3840 IU/mg. The LH potency for all preparations are compiled in Table 6 - I. From these results, unfortunately, it must be concluded that the precision for this assay system is very low, reflected in the high value of λ. (Assays with a λ-value of more than 0.4 are usually disregarded.) The reason is probably that the variation in maturity of the follicles is too large. *Channing & Ledwitz-Rigby (1975)* have shown that the LH-induced effect on cAMP undergoes a ten-fold increase when a medium-sized follicle passes into a large follicle. Although the follicles were carefully selected for uniform size when dissecting them, the boundary between "immature" and "mature" follicles is difficult to assess. Moreover, although only "immature" pigs were used, slaughterhouse material is by itself more heterogeneous than material from inbred animal strains. This point

was stressed by the finding of large fluctuations in the serum LH level of the immature slaughterhouse pigs.[1] One would expect to find LH to be hardly detectable in these animals but levels corresponding to the late follicular phase of the normal ovulatory cycle were frequently found.

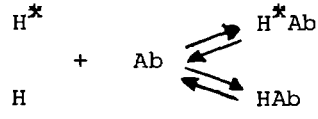
By comparison of the figures in Table 6 - I the LH_{II} preparations seem to be more potent than the LH_{I} preparations both from acetone-dried glands and from frozen glands. However, in view of the low precision of this method these differences cannot be regarded as significant.

1) Assays of these samples with a RIA-system for pLH were kindly carried out by Dr. D.F.M. van der Wiel, "Schoonoord" Research Institute for Animal Husbandry (I.V.O.), Zeist, the Netherlands.

7.1. Introduction

7.1.1. Principles

The radioimmunoassay (RIA) developed by *Yalow & Berson (1960, 1968)* and *Ekins (1960; Ekins, Newman & O'Riordan, 1968)* is based upon competition of radio-isotope-labelled (H^*) and unlabelled hormone (H) for binding sites at the hormone's specific antibody (Ab). After some time the following equilibrium exists:



The equilibrium lies far to the right. At equilibrium, H^* is either present as free hormone (F) or antibody-bound hormone (B). When the concentrations of H^* and Ab are kept constant, an increase in the concentration of H ($[H]$) in the reaction mixture results in a diminished number of antibody binding sites available for H^*, hence B will decrease and F will increase. Bound and free labelled hormone can be separated and the radioactivity measured. A standard curve can be constructed by plotting e.g. B/F as a function of $[H]$. Similarly the values of B and F for a sample containing an unknown amount of H can be determined and $[H]$ in the sample can then be computed from the standard curve. Underlying the radioimmunoassay is the assumption that the behaviour of standard and unknown

preparations in their competition with the labelled hormone for the antibody binding sites is identical.

7.1.2. Analysis of results

There are several ways of evaluating the counting results and constructing the standard curve. When both B and F have been determined the ratio B/F can be plotted against the concentration of unlabelled hormone ([H]). When either B or F has been counted and the initial total radioactivity of labelled hormone (T) is known, B/T or F/T as a percentage can be calculated and plotted against [H]. In all cases a hyperbola is the result. Because precise interpolation, necessary for the computation of the values for test samples, is troublesome in case of a non-linear standard curve, mathematical transformations have been applied. By plotting [H] on a logarithmic scale a sigmoidal curve is obtained, which shows a linear part. However, often this linear part is small, resulting in a small working range. *Rodbard & Lewald (1970)* suggested a logit/log transformation to straighten the whole line. (Logit A is given by $\ln \frac{A}{1-A}$). Logit B/B_0, where B is the fraction bound labelled hormone and B_0 is the value of B when no unlabelled hormone is present, plotted versus log [H] usually yields a straight line.

Concerning specificity of the radioimmunoassay the following must be observed. A protein hormone radioimmunoassay would only be 100% specific if the antibodies present would only recognize and react with intact molecules of the hormone

concerned. In practice this situation will never exist: any antiserum contains several populations of antibodies which react with contaminants of the hormone preparations; also, parts of the hormone molecules, containing pertinent antigenic determinants may have been split off, and will be determined as if they were intact molecules. The four glycoproteins, LH, FSH, TSH and hCG, present yet an additional problem because of their structural similarity within one species, especially with regard to their α-subunits. Antisera against a preparation of one of them will include antibodies directed against antigenic determinants situated on the α-chain. These antibodies will recognize also the α-subunit of each of the other three, causing aspecificity of the system. It has been possible to partly overcome this problem by developing systems based upon the so-called hormone specific subunit, i.e. the β-subunit. Such a system employs an antiserum raised against a purified β-subunit-preparation, a β-subunit-tracer, but a standard which may be an intact hormone preparation. In this type of system cross-reaction of any of the other glycoprotein hormones is minimal.

7.2. Procedure for the radioimmunoassays used in this investigation

7.2.1. Preparation of labelled hormone

Peptide hormones usually are labelled by incorporation of

radioactive iodine (^{125}I). The iodide is activated by oxidation and substituted ir the aromatic ring of tyrosine or histidine residues of the peptide. The peptide itself may be damaged by partial oxidation of certain groups, especially sulphydryl groups. This can be diminished by keeping the reaction time as short as possible. Besides the iodination damage there will be damage by radiation exerted by the incorporated iodine, e.g. alteration of the peptide structure. Therefore, the labelled hormone usually is subjected to a final purification step prior to use in an assay.

All chemicals were reagent grade purchased mainly from Merck (Darmstadt, Germany). All hormones were labelled by incorporation of ^{125}I (IMS-3, Amersham, U.K.), basically according to the method of *Hunter & Greenwood (1962)*. With the tube method described by *Brown & Reith (1967)* 5 µg hormone, dissolved in 20 µl 0.5 phosphate pH 7.4, 5 µg chloramine-T, dissolved in 5 µl 0.05 M phosphate pH 7.4 and 5 µl 0.5 M phosphate pH 7.4 were transferred into a glass tube containing 0.5 mCi Na ^{125}I in 5 µl NaOH-solution pH 8 - 11. The contents of the tube were mixed on a Vortex during 30 s. The reaction was terminated by adding 240 µg Na-metabisulphite in 100 µl 0.05 M phosphate pH 7.4 to neutralize the excess of chloramine-T. To prevent adsorption of the iodinated hormone to the glass during the next separation procedure 2 mg KI in 200 µl 0.02 M phosphate buffered saline pH 7.4 (PBS) containing 0.25% BSA (Povite, Amsterdam) was added. The labelled hormone was then separated from the other reactants on a small G 25 column (0.5 x 20 cm) previously equilibrated in 0.02 M PBS containing 0.25% BSA, and stored at -20°C. The labelled hormone was purified by

applying it on a Whatman C 11 cellulose column (0.5 x 20 cm) equilibrated in 0.02 M PBS. Labelled LH, LH_{α}, LH_{β} and TSH are adsorbed and, after washing the column with PBS to remove hormone fragments, are eluted with PBS containing 4% BSA. On the other hand, in the case of iodination of FSH the labelled hormone is not adsorbed and may be removed by washing with PBS. The following highly purified hormone preparations were used for labelling:

(1) hLH_{α} and hLH_{β} - prepared by dissociation of the LH preparation NM 04, which is type II LH.[1] The FSH content of these subunits is less than 1.25 IU 2nd IRP hMG/mg and their TSH content is less than 1.25 mIU 1st IRP TSH imm/mg, as determined by RIA. The contamination of β in hLH_{α} is 0.3% w/w, the contamination of α in hLH_{β} is 0.1% w/w.

(2) hFSH - CPDS/2, a preparation kindly donated by Dr. W.R. Butt, Birmingham, U.K., with a stated potency of 5200 IU 2nd IRP hMG/mg in the AOW *(Steelman & Pohley, 1953)*, an LH content of 1387 IU 2nd IRP hMG/mg measured by RIA and a TSH content of 0.3 IU 1st IRP TSH imm/mg.

(3) hTSH - NPA/2, immunochemical grade, a preparation kindly donated by the Hormone distribution officer, N.I.H., Bethesda, Md, U.S.A.

7.2.2. Preparation of antisera

Most peptide hormones are sufficiently immunogenic in several animal species, e.g. rabbit, guinea pig, sheep, goat etc. The immunogenicity can be enhanced by administering the hormone as an emulsion mixed with Freund's adjuvant. At regular intervals the antibody titer is determined. At a suitable time the animal is bled and its serum collected.

1) NM 04 was prepared by Closset and had properties similar to the LH preparation described by *Closset, Van Dalem, Hennen & Lequin (1975)*.

New Zealand white rabbits were used for the production of antisera as follows: Fifty µg of hormone was dissolved in 1 ml saline and emulsified with 1 ml complete Freund's adjuvant. The emulsion was injected intradermally divided over 20 spots on the back (Vaitukaitis & Robbins, *1971*). Once every two weeks during two months and once a week thereafter blood samples were obtained and the antibody titer determined. When the titer was at its maximum (after approx. 13 - 16 weeks), the animal was bled, the serum collected and stored at -70°C. Thus, the following antisera were prepared:

- code 9901 against nLH_I, preparation NIH 0708_I,
- code 8079 against nLH_{II}, preparation NIH 01;
- code 8082 against nLH_α, a preparation described in section 7.2.1.;
- code 8095 against hLH_β, a prepatation described in section 7.2.1.;
- code 8071 against pTSH, a preparation kindly donated by Dr. G. Hennen, Liège, Belgium.

In addition the following antisera were used: anti-FSH # 3 obtained from N.I.H., and anti-FSH "Pip", kindly donated by Dr. W.R.Butt, Birmingham, United Kingdom.

7.2.3. Standard preparations

As was mentioned before, it is essential that standard and test preparations are immunochemically identical. This requirement is best fulfilled when both preparations are obtained from comparable sources e.g. both from pituitary extracts, or both from urine etc. To be able to compare one's own results with those of other investigators the use of internationally accepted standard preparations is obligatory. Such preparations are

characterized by several criteria, e.g. biological acticity, physico-chemical properties, stability etc. As they are often available in only limited amounts, it is customary to use a local standard which has been calibrated against the international standard.

LH: Local standard - NM 01, prepared and described by *Closset et al (1975).* The potency of this preparation is 2256 IU 1st IRP LH imm/mg in the standard assay. All results are expressed in terms of the 1st IRP for immunoassay, formerly known as MRC 68/40 with an assigned potency of 77 IU/ampoule (approx. 6640 IU/mg) *(WHO - ECBS 26th Report, 1975).*

FSH: Local standard - CPDS/6, a preparation kindly dorated by Dr. W.R.Butt, Birmingham, U.K., with a stated potency of 5600 IU 2nd IRP hMG/mg in the AOW and an LH content of 119 IU 1st IRP LH imm/mg; TSH content not known. All results are expressed in terms of the 2nd IRP hMG with an assigned potency of 40 IU/ampoule (approx. 9 IU/mg) *(WHO - FCBS 15th Report, 1964).*

TSH: No local standard was used. All results are expressed in terms of the 1st IRP TSH for immunoassay, formerly known as MRC 68/38 with an assigned potency of 0.15 IU/ampoule (approx. 3.2 IU/mg) *(WHO - ECBS 26th Report, 1975).*

7.2.4. Separation of bound and free hormone fractions

Many procedures are available, the choice depending on whether both the bound and free labelled hormone are to be measured or only one of them. The most common methods comprise precipitation of fraction B by polyethyleneglycol or ammonium sulphate, immunological precipitation of B by a second anti-

body, or adsorption of F to dextran-coated charcoal. For extended surveys the reader is referred to *Yalow & Berson (1973)* and *Ratcliffe (1974)*.

Our separation was obtained by immunological precipitation of the bound fraction with a sheep anti-rabbit γ-globulin coupled to cellulose (DASP, Organon).

7.2.5. Assay procedure

All components were dissolved in or diluted with PBS pH 7.4 containing 1% BSA. The incubation mixture, pipetted in disposable glass tubes (Rhesus ampoules, Forma Vitrum, St. Gallen, Switzerland) consisted of 50 µl of labelled hormone, 50 µl of antiserum diluted as appropriate, 400 µl of the PBS containing the standard or test preparation. Incubation was carried out either during 2 h. at 37°C (hereafter referred to as quick assays) or during 4 days at 4°C (hereafter referred to as standard assays). At the end of the incubation 1 ml of a 1:10 dilution of DASP was added to each tube. After mixing, the tubes were incubated for another 30 - 180 min. depending on the binding capacity of the particular batch of DASP. The tubes were centrifuged, 2 min. at 2000 g, the supernatant siphoned off and the pellet washed twice with saline. Finally, the radioactivity in the pellet was counted in an LKB Wallac 80000 gammascintillation counter. The data were evaluated using a computer programme developed by *Rodbard & Lewald (1970)*.

7.2.6. Quick assays

During purification, hormones should be kept in solution as short as

Table 7 - I. Summary of various radioimmunoassay systems used for determination of human LH, FSH and TSH activities.

Hormone	Labelled hormone preparation	Antiserum (final dilution)		Local standard preparation	International standard preparation	Mode of assay (standard or quick)
LH	NM 04 LH_{β} 20000 cpm ∿ 500 pg	8095	1:10000	NM 01	1^{st}IRP LH imm	quick
LH	NM 04 LH_{β} 5000 cpm ∿ 100 pg	8095	1:100000	NM 01	1^{st}IRP LH imm	standard
LH	NM 04 LH_{α} 5000 cpm ∿ 100 pg	8082	1:100000	NM 01	1^{st}IRP LH imm	standard
FSH	CPDS/2 FSH 20000 cpm ∿ 500 pg	Pip	1:10000	CPDS/6	2^{nd}IRP hMG	quick
FSH	CPDS/2 FSH 5000 cpm ∿ 100 pg	NIH #3	1:100000	---	2^{nd}IRP hMG	standard
TSH	NPA 2 TSH 5000 cpm ∿ 100 pg	8071	1:25000	---	1^{st}IRP TSH imm	standard

possible for reasons of stability. Monitoring the elution profiles of the gelfiltration columns (chapter 6) by standard RIA-procedure would take at least 5 - 7 days. Therefore, quick assays were developed which differed from the standard procedure in that a larger amount of labelled hormone was employed to reduce the counting time, while a decreased dilution of the antiserum was used and incubation done at 37°C to reach equilibrium faster. In this fashion the results were known within 2 days.
All radioimmunoassay systems used are summarized in Table 7 - I.

7.3. Results and discussion

LH. The immunological LH activity of all preparations was measured by the quick as well as the standard LH_α-system. Table 7 - II shows the potencies in terms of the local standard NM 01 and the international standard 1st IRP LH imm. The index of precision λ usually varied between 0.02 and 0.08, which is the normal range in this laboratory. As this Table shows, for all batches analysed the LH_I preparations have a lower potency than the LH_{II} preparations both in the quick and in the standard assay. There could be several explanations for this phenomenon:

(1) It was shown by *Reguin, Rogers, Closset & Hennen (1971)* that in the standard LH_β-system highly purified LH_β is about 10 times as potent as NM 01 (an LH_{II} preparation formerly called LH/LN) on a weight basis (Fig. 7 - 1). One might assume then, that free LH_β, present as contamination in the LH preparations, would affect the potency proportionally to the

Table 7 - II. Potencies of human LH_I and LH_{II} determined by radioimmunoassay.

Batch[1]	LH_I		LH_{II}	
	NM 01[2] (mean; 95% fiducial limits)	IU 1st IRP LH imm/mg (mean; 95% fiducial limits)	NM 01[2] (mean; 95% fiducial limits)	IU 1st IRP LH imm/mg (mean; 95% fiducial limits)
	Standard system			
NM 07	1.00 (0.92-1.10)	2660 (2440-2920)	1.05 (0.91-1.23)	2790 (2400-3280)
08			0.73 (0.60-0.88)	1940 (1590-2340)
09	0.43 (0.36-0.52)	1140 (960-1380)	1.00 (0.88-1.16)	2660 (2340-3100)
14	1.00 (0.95-1.17)	2660 (2520-3120)	1.42 (1.23-1.65)	3770 (3270-3480)
15	0.63 (0.51-0.78)	1670 (1350-2080)	1.45 (1.19-1.72)	3850 (3160-4570)
16	0.65 (0.52-0.79)	1730 (1380-2100)	1.12 (0.81-1.68)	2980 (2150-4460)
10	0.31 (0.21-0.39)	830 (560-1040)	0.45 (0.39-0.53)	1200 (1040-1410)
11	0.27 (0.20-0.36)	720 (530- 960)	0.96 (0.65-1.45)	2550 (1730-3850)
	Combination 07-16:	1900 (1670-2210)	Combination 07-16:	3000 (2790-3300)
	10-11:	810 (750- 890)	10-11:	2040 (1460-2680)
	Quick system			
NM 07	0.63 (0.56-0.78)	4180 (3720-5180)	0.86 (0.82-0.90)	5710 (5440-5980)
08			0.98 (0.93-1.01)	6510 (6180-6710)
09	0.48 (0.43-0.53)	3190 (2860-3520)	1.00 (0.97-1.03)	6640 (6440-6840)
14	0.59 (0.53-0.65)	3920 (3520-4320)	1.00 (0.96-1.03)	6640 (6370-6840)
15	0.34 (0.31-0.37)	2260 (2060-2460)	1.13 (1.08-1.20)	7500 (7170-7970)
16	0.40 (0.38-0.42)	2660 (2520-2790)	1.30 (1.24-1.36)	8630 (8230-9030)
10	0.33 (0.32-0.36)	2190 (2120-2390)	0.43 (0.41-0.45)	2860 (2720-2990)
11	0.25 (0.23-0.27)	1660 (1530-1790)	0.35 (0.33-0.37)	2320 (2190-2460)
	Combination 07-16:	3240 (2910-3620)	Combination 07-16:	6600 (6350-6880)
	10-11:	1930 (1700-2190)	10-11:	2590 (2340-2850)

1) NM 07 - NM 16: acetone-dried glands; NM 10 - NM 11: frozen glands.
2) NM 01 (IU 1st IRP LH imm/mg): 2656 (Standard system); 6640 (Quick system); see text for details.

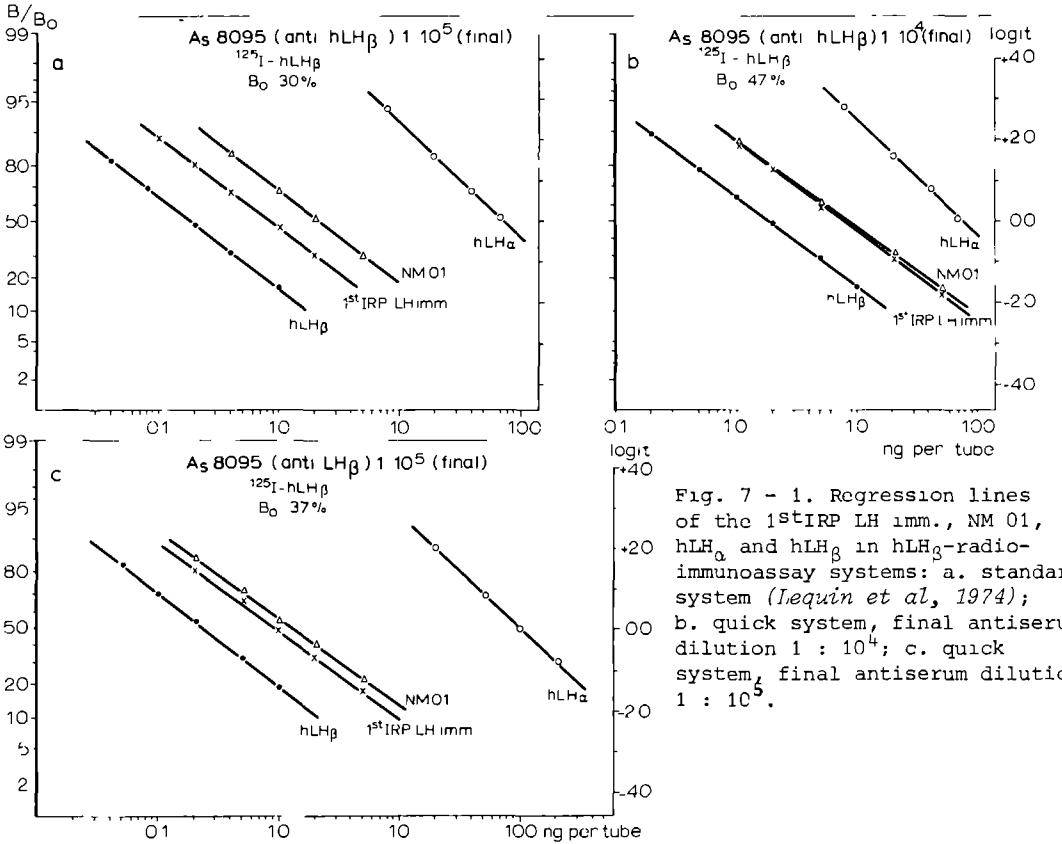


Fig. 7 - 1. Regression lines of the 1stIRP LH imm., NM 01, hLH_{α} and hLH_{β} in hLH_{β}-radioimmunoassay systems: a. standard system *(Lequin et al, 1974)*; b. quick system, final antiserum dilution 1 : 10^4; c. quick system, final antiserum dilution 1 : 10^5.

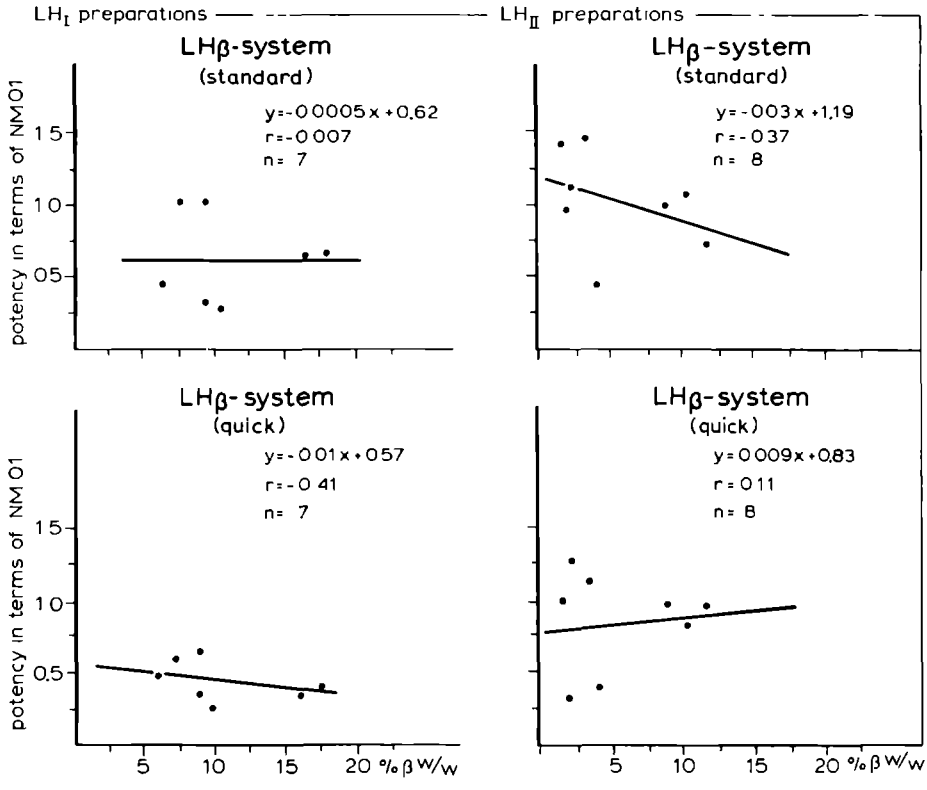


Fig. 7 - 2. Relationship between the percentage LH_β-subunitcontamination and the potency in terms of NM 01 of the LH_I and LH_{II} preparations measured in the standard and quick LH_β-radioimmunoassay systems.

degree of contamination. In Fig. 7 - 2 the potencies of the LH preparations in terms of NM 01 have been plotted against the LH_β contaminations that were reported in Table 3 - III. It appears that there is no correlation and therefore the above possibility must be rejected.

(2) The LH_β-systems employ antibodies raised against a β-subunit preparation derived from NM 04 LH_{II} as well as an LH_{II}-β-subunit-tracer (a so-called homologous system). It would be

conceivable that intact LH_I-molecules are less able to compete in this system than intact LH_{II}-molecules. A control experiment using a system based upon the β-subunit from LH_I would have been appropriate. Unfortunately, at the time such a system was not available. However, the answer to the question could be approximated by comparison of the behaviour of LH_I and LH_{II} preparations in homologous intact LH_I- and intact LH_{II}-systems as well as cross-over experiments. The following four combinations were run simultaneously: 1. Anti-LH_I + tracer LH_I; 2. Anti-LH_I + tracer LH_{II}; 3. Anti-LH_{II} + tracer LH_I; 4. Anti-LH_{II} + tracer LH_{II}. In all these systems the activities of an LH_I preparation (NM 0708_I) and an LH_{II} preparation (NM 01_{II}) were compared. The dose-response lines obtained are presented in Fig. 7 - 3;those for NM 0708_I are not straight over the whole range probably due to cross-reacting components. The difference in activities of the two preparations appears to be the same in all four systems and is similar to that found in the standard LH_{II}β-system (Table 7 - II), i.e. NM 0708_I/NM 01_{II} ∿ 0.8 - 1.0. Therefore, it would seem unlikely that the observed difference in potency between LH_I and LH_{II} is the consequence of using an LH_{II}β-system. Since the difference in immunological potencies of LH_I and LH_{II} preparations cannot be attributed to methodological influences it must be concluded that the LH_I preparations in immunological respect just are slightly less purified than the LH_{II} preparations.

Comparison of the results of quick and standard assays is of interest, if only for rating the value of the quick assay.

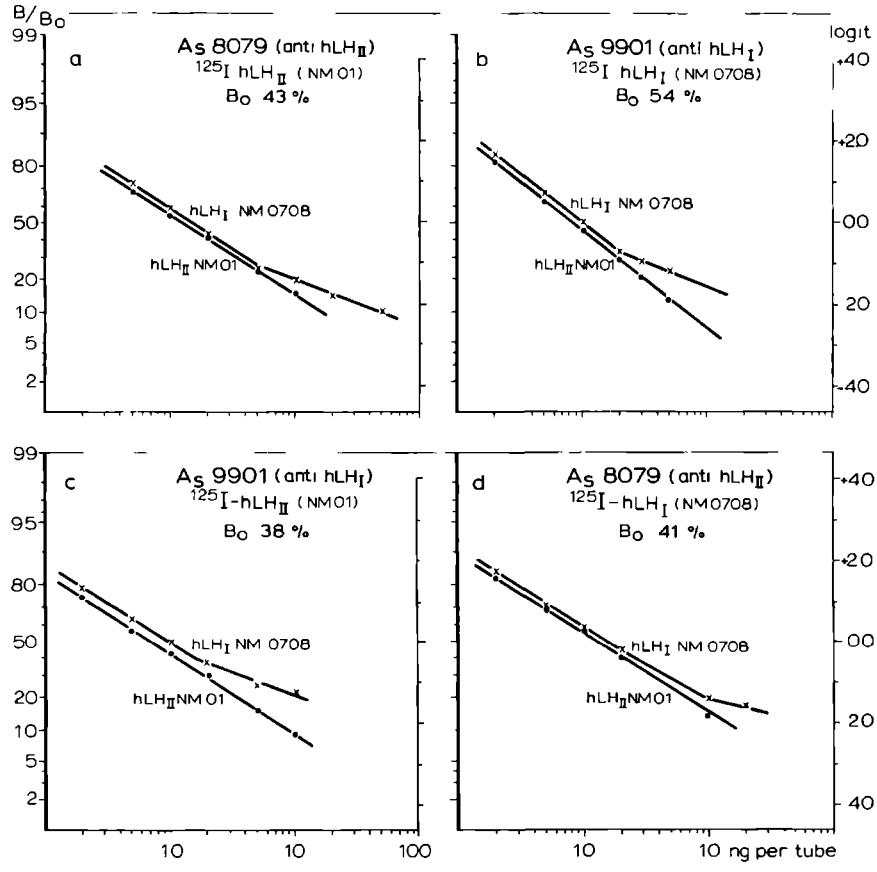


Fig. 7 - 3. Cross-over experiments, with exchanges of antisera directed against LH_I and LH_{II}, and labelled hormones prepared from LH_I and LH_{II}, designed to elucidate whether there is a relationship between these assay components and the relative potency of LH_I and LH_{II} preparations in the intact LH radioimmunoassay system.

At first glance all preparations seem to have roughly the same potencies in quick and standard assays, at least when expressed in terms of the local standard NM 01 (Table 7 - II). However, comparing the results of quick and standard assays when these are expressed in units of the 1st IRP LH imm., considerable

differences in potency become apparent. *Lequin et al (1974)* have established that the potencies of highly purified LH_β, 1st IRP LH imm. and NM 01 in a standard LH_β-system were in a ratio of 10 : 2.5 : 1 (Fig. 7 - 1a). As the potency of the 1st IRP LH imm. has been assigned to be 6640 IU/mg, it follows that the potency of NM 01 in this LH_β-system is 6640 x 1/2.5 = 2656 IU/mg. The same preparations were tested in the quick LH_3-system. This time the potencies were in ratio of 4 : 1 : 1 (Fig. 7 - 1b). Since the antiserum dilution used in the quick system was a factor 10 less than in the standard system, this might have influenced the relative potencies. A control experiment using the same antiserum dilution in the quick system that was used in the standard system, showed that the relative potencies remained 4 : 1 : 1 (Fig. 7 - 1c). Thus, in the quick system the 1st IRP LH imm. and NM 01 are equipotent (6640 IU/mg). How can the difference in potency of NM 01 between quick and standard system be explained? It has been shown before *(Lequin, 1976)* that the 1st IRP LH imm. contains approx. 26% β-subunits (w/w), and NM 01 approx. 3%. While these preparations have been kept lyophilized at -20°C for several years there has been no change in these figures. However, recent observations in this laboratory indicate that, when in solution at elevated temperature (37°C), the preparations are not stable and some dissociation into subunits occurs. Since the components of the assay mixture are the same in quick and standard assays but the only difference concerns the temperature, the observed difference in potency could well be attributed to the tem-

perature factor. Furthermore, there appears to be a slight negative correlation between the degree of temperature-dependent dissociation and the amount of subunits already present, since the potency of the 1st IRP LH imm. with a high initial content of β-subunits does not noticeably shift with respect to that of pure LH_β whereas the potency of NM 01 with a low content of β-subunits, alters drastically. From the fact that the potencies of the LH preparations, when measured against NM 01, hardly show differences between the standard and quick systems, it may be concluded that these preparations shift more or less in a manner similar to that of NM 01.

Finally, comparison of the results of the preparations from frozen glands and from acetone-dried glands show that irrespective of system and standard, the preparations from frozen glands (NM 10 - 11) have a lower specific immunological activity, probably due to a larger content of inert protein.

FSH and TSH. The FSH- and TSH-contaminations in the LH preparations were measured in the standard assay systems specific for these hormones. The results are presented in Table 7 - III. The FSH-contamination in the LH_I preparations is higher than in the LH_{II} preparations. This is not surprising when it is realised that complete separation of FSH and LH_I was not possible *(chapter 2)*. These figures for the FSH-contamination are in agreement with those found by *in vivo* bioassay *(chapter 5)*. As there was no bioassay available for measuring TSH activity, these figures cannot be checked, but,

Table 7 - III. Determination of FSH and TSH contaminations in human LH_I and LH_{II} by radioimmunoassay.

	LH_I		LH_{II}	
	FSH	TSH	FSH	TSH
Batch[1]	IU 2nd IRP/mg (mean; 95% fiducial limits)	IU 1st IRP TSH imm/mg (mean; 95% fiducial limits)	IU 2nd IRP/mg (mean; 95% fiducial limits)	IU 1st IRP TSH imm/mg (mean; 95% fiducial limits)
NM 07	22 (17-27)	0.035 (0.033-0.037)	24 (19-29)	0.191 (0.171-0.215)
08			7 (6- 9)	0.030 (0.024-0.036)
09	20 (15-26)	0.250 (0.231-0.275)	14 (11-17)	0.042 (0.034-0.050)
14	11 (8-14)	0.001 (0.000-0.002)	12 (10-14)	0.031 (0.025-0.035)
15	88 (78-98)	0.039 (0.037-0.042)	8 (7-10)	0.012 (0.010-0.014)
16	43 (35-53)	0.057 (0.052-0.062)	4 (3- 5)	0.022 (0.018-0.026)
10	56 (48-64)	0.005 (0.004-0.006)	9 (8-11)	0.005 (0.004-0.006)
11	39 (32-46)	0.008 (0.007-0.009)	5 (4- 6)	0.001 (0.000-0.002)

1) NM 07 - NM 16: acetone-dried glands; NM 10 - NM 11: frozen glands.

if the assumption is made that the immunological activity in some way represents the biological activity, at least they form a strong indication that the LH preparations are contaminated only very slightly with TSH. This impression is strengthened when the TSH activities of these LH preparations and a highly purified TSH preparation, e.g. the 1st IRP TSH imm., are compared: the difference is approximately a hundredfold.

8.1. Introduction

8.1.1. Principles

Although during the last decade the radioimmunoassay was accepted as the method of choice for estimating very low concentrations of circulating peptide hormones, there was an increasing recognition that this assay system is not only measuring the "active" hormone, i.e. those molecules which are able to elicit a physicological response from the target organ. Also "inactive" fragments are recognized by the antibody-binding sites. Autoradiographic studies indicated that a peptide hormone is concentrated in its target organ where it is bound to the cell membrane *(Espelord, Vaftolin & Paulsen, 1968, Eshkol & Lunerfeld, 1969, Midgley, 1973)*. *Catrecasas (1969)* coupled insulin to Sepharose and incubated this complex with isolated fat cells; the fact that the solid-phase insulin which could not penetrate into the cells, was able to exert its normal biological effects led him to the conclusion that it was sufficient for the hormone to be in contact with the plasma membrane of the cells. From studies concerning binding of labelled ACTH to extracts of an adrenal tumor *Lefkowitz, Roth, Pricer & Pastan (1970a,b)* developed the first radioligand-cell-membrane-receptor assay (RLA). This assay is based upon the principle of competition between labelled and unlabelled hormone for binding sites (receptors) on the plasma

membranes of target organ cells. It is assumed that the hormone-receptor complex is identical to that existent *in vivo* and that only the "biologically active" hormone molecules are measured. Since 1970, radioligand assay systems have been developed for a large number of protein and peptide hormones; only those for LH (or hCG) will be discussed in this chapter.

8.1.2. Analysis of results

Again, there is a close conformity between RLA and RIA because the methods for statistical evaluation of the results developed by *Rodbard & Lewald (1970)* for the RIA as well as the theoretical considerations about reliability *(Borth, 1957)* are also applicable to the RLA *(Rodbard, 1975)*. With regard to one of the reliability criteria namely specificity, however, there are some important differences. Natural receptor sites, unlike induced antibodies, bind the hormone concerned more specifically. This has been confirmed in studies investigating cross-reactivity of other highly purified hormone preparations. Further, in the case of the glycoproteins in the RLA, there is no interference by structurally similar subunits, because the subunits are completely inactive.

8.2. Procedure for the radioligand assays used in this investigation

8.2.1. Preparation of labelled hormone

The binding of the hormone to its tissue binding sites is much

more susceptible to alterations in the hormone structure than is the binding to antibody-binding sites. This means that during the labelling reaction mild conditions have to be observed. Because incorporation of 125-iodine involves an oxidative milieu, some investigators have turned their attention to the use of tritium. This isotope may be incorporated by e.g. methylation or carbamylation of the protein chain *(De la Llosa, Durosay, Tertin-Clary & Jurisz, 1974; De la Llosa-Hermier, Hermier & De la Llosa, 1976)* or by oxidation and reduction of the sialic acid *(Vaitukaitis, Hammond, Ross, Hickman & Ashwell, 1971a; Vaitukaitis, Sherins, Ross, Hickman & Ashwell, 1971b)*. Besides the fact that the tertiary structure of the hormone is less affected by incorporation of tritium than by iodine, another advantage of tritium over 125-iodine is the longer shelf-life of the tritiated hormone, due to a longer decay-time and a less damaging radiation effect on the molecular structure. Unfortunately, however, important disadvantages of tritium are that it is difficult to incorporate and that it is a β-emitter, which necessitates the use of scintillation fluid and long counting-times. Therefore, most investigators still choose 125-iodine as a label and modify the conditions of the iodination reaction to keep the damage as low as possible. Nowadays, the chloramine-T method *(Hunter & Greenwood, 1962)* under mild conditions as described by *Leidenberger & Reichert (1972b)* and the enzymatic lactoperoxidase method developed by *Miyachi, Chrambach, Mecklenburg & Lipsett (1973)* are most commonly in use, but other gentle

iodination procedures have been described as well *(Butt, 1972)*.

Since the chloramine-T method under mild conditions *(Leidenberger & Reichert, 1972b)* as well as the lactoperoxidase method *(Miyachi et al, 1973)* have been recommended, it was decided to compare these two methods with respect to the *in vivo* biological activity of the labelled products. The chloramine-T method was carried out as described in chapter 7 section 7.2.1. except that the amount of chloramine-T was reduced to 2.5 µg, and the reaction mixture was kept at 0°C. The lactoperoxidase method, modified by *Van Toorenenbergen & Lequin*[1], was used: 5 µg LH, dissolved in 15 µl 0.05 M phosphate pH 7.4, 1.25 µg lactoperoxidase coupled to Sepharose 4B (to facilitate its removal from the reaction mixture) suspended in 15 µl 0.01 phosphate buffered saline pH 6.5 and 5 µl 0.4 mM H_2O_2 were pipetted into a glass tube containing 0.5 mCi ^{125}I in 5 µl NaOH. The tube was placed in an ice bath and the contents were mixed. After 15 minutes the reaction was terminated by adding 200 µl 0.025 M NaN_3 + 0.025 M KI. The labelled hormone was separated from the other reactants on a small G 25 column (0.5 x 20 cm) previously equilibrated in 0.02 M PBS containing 0.25% BSA and stored at -20°C. The *in vivo* biological activity of the labelled hormones was determined in Parlow's OAAD-test *(Parlow, 1961; see chapter 5, section 5.3)*. The results of this study, summarized in Table 8 - I, indicate that both methods produced equivalent results, concerning the biological activity as well as the specific incorporated radioactivity. The chloramine-T method[1] was chosen because it is the easiest to perform. For labelling the LH_{II} preparation NM 09 was

1) Van Toorenenbergen A.W. & Lequin R.M., unpublished results.

used. Before use the tracer was purified as described in chapter 7 section 7.2.1.

Table 8 - I. Effect of two iodination methods on the biological activity (OAAD-test) of human LH_{II}

	Specific activity µCi/µg	Final biol. act. / Initial biol. act. in %	λ
Lactoperoxidase	40	97 (48-193)	0.33
	24	51 (33- 80)	0.16
	15	64 (40-112)	0.30
Chloramine-T	23	75 (62- 92)	0.20
	34	80 (47-150)	0.65
	33	190 (62-497)	0.65

8.2.2. Preparation of binding tissue

Since both the ovary and the testis are target organs for LH, either one may be selected for the preparation of tissue fragments. Methods employing the whole organ after fragmentation *(Leidenberger & Peichert, 1972a,b; Lee & Ryan, 1971, 1972; Tsuruhara, Van Hall, Dufau & Catt, 1972, Koch, Zor, Grobsieng, Larprecht, Pomerantz & Lindner, 1974)* as well as techniques involving isolated specific cell types *(Catt, Dufau, Tsuruhara, 1971, 1972; Kammerman, Canfield, Kolena & Channing, 1972)* have been reported.

Binding tissue was prepared according to *Koch et al (1974)*. Female Wistar rats were rendered pseudo-pregnant by pretreatment with PMS (50 IU) +

hCG (25 IU). Between 5 and 9 days after the hCG injection the animals were killed. The ovaries were carefully dissected out and weighed. The ovarian weight varied from 60 - 90 mg. The ovaries were homogenized at $0^{o}C$ in a Krebs Ringer bicarbonate buffer pH 7.4, containing 0.1% BSA (Sigma) and 0.2% glucose (KRBGA buffer), using an all-glass homogenizer with a loose-fitted pestle, manually applying three strokes only. The homogenate containing intact cells as well as membrane-fragments was strained through cheese cloth and diluted with the same buffer to contain an equivalent of 10 mg ovarian weight per ml.

8.2.3. Standard preparations

Essentially the same stipulations that were stated for preparations measured in the RIA *(chapter 7 section 7.2.3.)* must also be fulfilled in the RLA, i.e. identical behaviour of standard and test preparations, comparable sources and calibration against international standards.

As local standard in each assay NM 14_{II} was used. When calibrated against the 1^{st} IRP for LH and FSH for bioassay, formerly known as MRC 69/104, with an assigned potency of 25 IU LH/ampoule, NM 14_{II} turned out to have a potency of 21400 IU/mg.(When calibrated against the 2^{nd} IRP hMG, NM 14_{II} had a potency of 23000 IU/mg.)

8.2.4. Assay procedure

All components were dissolved in or diluted with KRBGA buffer. The incubation mixture, pipetted in round-bottommed disposable glass tubes

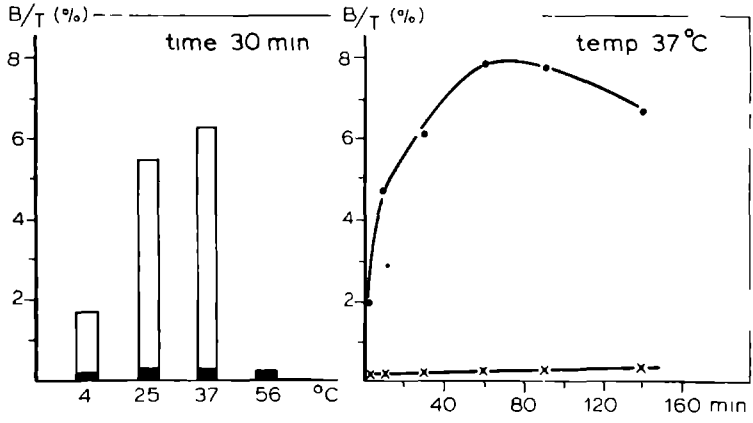


Fig. 8 - 1. Determination of optimal conditions for the procedure of the radioligand assay for LH. *Left.* Influence of incubation temperature on binding. Open bars indicate percentage total binding, closed bars indicate percentage aspecific binding. *Right.* Influence of incubation time on binding. ●—● indicates percentage total binding, x—x indicates percentage aspecific binding.

(6 x 50 mm), consisted of 1.0 ml of tissue homogenate, 50 µl of labelled hormone and 200 µl of KRBGA buffer containing standard or test preparation. The incubation was carried out during 1 h. at 37°C, which appeared to be the optimal condition (Fig. 8 - 1), under a 95% O_2 + 5% CO_2 atmosphere. Under these circumstances the total binding ranged from 5 - 10% and the non-specific binding, defined as the binding in the presence of an infinite amount of unlabelled LH or hCG, from 0.1 - 0.5%. At the end of the incubation the tubes were centrifuged 5 min. at 2000 g, the supernatant siphoned off and the pellet was counted in an LKB Wallac 80000 gamma scintillation counter. To ascertain that labelled and unlabelled hormone behaved identically in the competition for binding sites, the following experiment was done. By adding increasing amounts of tracer a competition between the labelled molecules themselves was generated, the "standard" curve obtained in this manner parallels the normal standard curve (Fig.8-2).

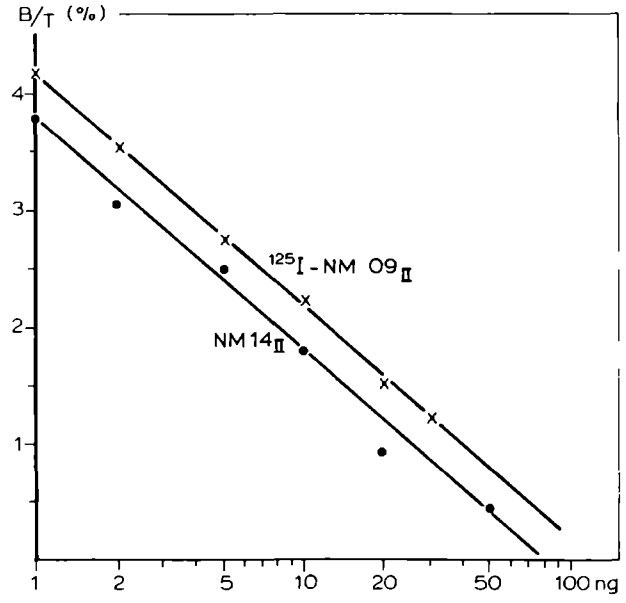


Fig. 8 - 2. Regression lines for radioactive labelled and unlabelled LH in the radioligand assay. Specific activity ^{125}I-NM 09_{II}: 30 µCi/µg.

For the calculation of the potencies of the LH preparations the data were evaluated using either a computer programme developed by *Podbard & Lewald (1970)* or a computer programme based upon the parallel-line method by *Brownlee (1960)*. The results of several assays were combined using a computer programme based upon a method by *Finney (1964)*. The last two computer programmes were kindly made available by Dr. W. Bridson (N.I.H., Bethesda, Md. U.S.A.).

8.3. Results and discussion

The potencies of the LH preparations tested in the RIA are expressed in terms of the local standard NM 14_{II} and the inter-

national reference preparation 1st IRP LH/FSH bio. The choice of this particular international reference preparation needs some explanation. The 26th Committee of the WHO recommended to make a distinction in the use of standards for bioassays and for immunoassays *(WHO - FCRS 26th Report, 1975)*. Henceforth, it established International Reference Preparations and International Standards especially for the purpose of bioassays, and others especially for the purpose of immunoassays. However, there is no agreement whether radioligand assays belong to the bioassays or to the immunoassays. On the one hand it can be argued that, like the RIA, the RLA measures hormone levels on the basis of the hormone structure rather than on the basis of hormone function; on the other hand it employs tissue (cells, membranes) of target organs and could, therefore, be considered to be a bioassay. Although the 1st IRP LH/FSH bio recently was established for use in bioassays, this preparation (formerly MRC 69/104) has been prepared from LER 907, a well-known pituitary preparation that is still widely used as reference preparation for radioimmunoassay. Also in view of the decision of the 26th Committee to recommend the 1st IRP LH/FSH bio as a provisional standard for radioimmunoassay of FSH, this preparation was selected here for use in the RLA by lack of a good alternative.

Table 8 - II shows the potencies of all LH preparations tested. These results show that this type of assay has a lower precision (reflected in a higher λ-value) than the RIA. Also, for some preparations (e.g. NM 0708_I, NM 15_{II}), repeated

Table 8 - II. Potencies of human LH_I and LH_{II} determined by radioligand assay.

Batch[1]	LH_I IU 1st IRP LH/FSH bio/mg (mean; 95% fiducial limits)		λ	LH_{II} IU 1st IRP LH/FSH bio/mg (mean; 95% fiducial limits)		λ
NM 07	9400 (7900-11300)	1300 (11300-15200)	0.09	13900 (10500-34200)	15000 (13300-17100)	0.11
	6800 (3600- 7500)		0.14	19900 (17100-23800)		0.09
	17500 (13500-21900)		0.16	11800 (9600-14800)		0.10
08	25900 (19500-34500)		0.14	5300 (2800- 9000)	6600 (5100- 8100)	0.25
				4900 (3900- 6400)		0.13
				8800 (5600-12600)		0.19
09	3400 (2800- 4000)	5100 (4700- 6000)	0.12	21400 (19000-23800)	22300 (19900-25300)	0.06
	6200 (4500- 8300)		0.09	22000 (18800-26300)		0.08
	7300 (5100- 9200)		0.09	28000 (20100-38710)		0.15
				20100 (16300-24000)		0.20
14	3200 (2800- 3900)	3400 (2600- 4300)	0.10	22000 (17500-27300)	21400 (17600-25700)	0.09
	3900 (2100- 5100)		0.28	20800 (16800-24100)		0.08
				21200 (18000-23800)		
15	4700 (2600- 9400)	4900 (2400- 9800)	0.10	28900 (24800-33800)	17100 (15200-19300)	0.09
	5400 (2100-10300)		0.12	11300 (9400-13700)		0.07
16	9800 (8800-13500)	5400 (4500- 6000)	0.09	20100 (17100-23500)	19000 (17100-21400)	0.08
	4100 (3000- 4900)		0.11	18400 (14600-21600)		0.07
10	4900 (3600- 6000)	5100 (4000- 6400)	0.11	11100 (8300-16700)	15200 (12600-18600)	0.12
	5600 (2400- 4700)		0.13	19300 (15400-24400)		0.09
11	2800 (1500- 3400)	4100 (3600- 4700)	0.08	8100 (6000-10700)	7900 (7300- 9800)	0.10
	5600 (4500- 6800)		0.11	7700 (6000- 8800)		0.07
	Combination 07-16:	6200 (5700- 6800)		Combination 07-16:	17400 (16300-19000)	
	10-11:	4800 (4200- 5500)		10-11:	12000 (10300-13200)	

1) NM 07 - NM 16: acetone-dried glands; NM 10 - NM 11: frozen glands.

potency determinations by the RIA yield rather divergent results. It is difficult to explain fully this last variability. Buffer, hormone solutions and tracer were always prepared freshly. The only variable was the day during pseudo-pregnancy on which the ovaries were used, i.e. the period of time elapsed after the pretreatment of the rats with PMS and hCG. From pilot studies concerning the effect of the length of pseudo-pregnancy on the sensitivity of the OAAD-test *(chapter 5 section 5.3,)* and from the reports by *Parlow (1961)* and *Koch et al (1974)* it may be inferred that the condition of pseudo-pregnancy was maintained constant during a number of days. However, the possibility of obscured day-to-day variations, e.g. in ovarian weight,condition of binding sites, which might have caused differences in the tissue preparations used cannot be excluded.

From Table 8 - II it can be seen that the potency of LH_I preparations is lower than that of LH_{II} preparations. This could be explained by the fact that an LH_{II} preparation was used as tracer and it would be conceivable that LH_I molecules are less able than LH_{II} molecules to compete with labelled LH_{II} molecules for binding sites. Therefore, a control experiment was done in which an LH_I preparation was used as labelled hormone (Fig. 8 - 3). It appears that the difference in potency is not due to the type of LH used for labelling. Although it has been shown in Table 3 - III that the LH preparations show a considerable variation in subunit contamination, this cannot be the explanation for the observed

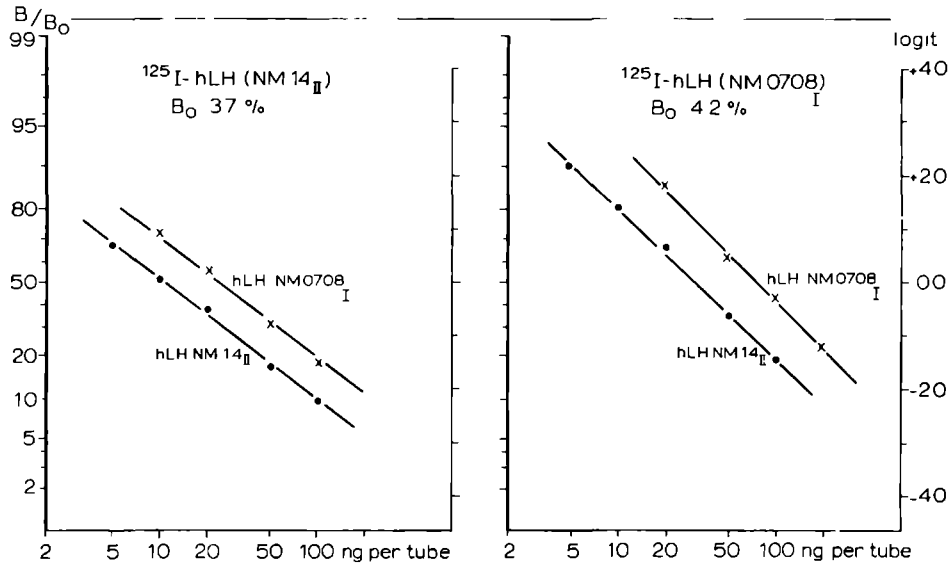


Fig. 8 - 3. Relationship between the type of LH used for labelling and the relative potencies of LH_I and LH_{II} in the radioligand assay.

potency differences because a pertinent control study showed the subunits to be totally inactive in the RLA. This last result confirms similar conclusions by other investigators *(Kammerman et al, 1972; Catt, Dufau & Tsuruhara, 1973)*.

Finally, the sialic acid content, which is of great importance for the *in vivo* biological activity, probably has also some influence, since it was shown that asialo-hCG was more active in the RLA than the native hormone *(Tsuruhara et al, 1972; Kammerman et al, 1972; Lequin et al, 1975)*. Because the LH_{II} preparations have a lower sialic acid content than the LH_I preparations (Table 3 - I) this might explain that the potency of the LH_{II} preparations is higher. The last argument, however, does not hold for the preparations

from frozen glands (NM 10, 11) because no differences in sialic acid could be shown to exist. Concerning these batches there is yet another point. Comparison of the results of the batches NM 10 and 11 (frozen glands) with those of the other batches (acetone-dried glands) shows, both for LH_I and for LH_{II}, that the former have a lower potency, probably because they still contain an amount of inert protein.

Purification of hormones to a high degree and subsequent determination of the physico-chemical properties is compulsory for a complete understanding of their activity. It is, therefore, essential to remove contaminating hormones in order to avoid biases. Furthermore highly purified hormone preparations are necessary for developing assay methods e.g. for routine clinical purposes. The crucial questions are: (1) What do we mean by a highly purified hormone preparation, and (2) Which other activities are exerted by such preparations that are selected on the basis of *in vivo* biological activity. Trying to find an answer to these questions for human pituitary luteinizing hormone was the main purpose of this investigation. It appeared possible to isolate and purify LH from crude glycoprotein material prepared from acetone-dried pituitary glands and from frozen pituitary glands. Besides the bulk fraction of LH isolated according to conventional methods a second fraction of LH, originally regarded as a contamination of the FSH fraction *(Reichert, 1967; Saxena & Rathnam, 1968; Butt, Crooke & Wolf, 1965; Graesslin, Weise & Bettendorf, 1972)* could be isolated in substantial quantities and turned out to be another form of LH. Both LH fractions, further referred to as LH_{II} and LH_{I} respectively, were almost completely free from the other pituitary glycoprotein hormones, namely FSH, as assessed by bioassay and radioimmunoassay, and TSH, as assessed by radioimmunoassay. The first question could in the first instance be answered as follows: a highly purified

hormone preparation is one that contains not more than negligable quantities of other hormones. In the purification of any protein one tries to remove selectively as much inert protein as possible, the success of which is reflected in the values for the specific activity after each step. Since a hormone is identified by its *in vivo* biological activity, in hormone purification this type of activity is the basis for the above-mentioned purification. For most hormones the purification is regarded as completed when the specific biological activity cannot further be increased, i.e. when it reaches a plateau-value. However, in the case of glycoproteins the situation is more complicated. For these hormones the *in vivo* biological activity is closely correlated to the content of the terminal carbohydrate, sialic acid *(Van Hell, Matthijsen & Homan, 1968; Goverde, Veenkamp & Homan, 1968; Schuurs, De Jager & Homan, 1968; Van Hall, Vaitukaitis, Ross, Hickman & Ashwell, 1971)*. Due to their proneness to loose part of the initial sialic acid content during purification, the theoretically maximal specific biological activity, which is probably higher than the plateau-value, cannot be known for these hormones. As a consequence it is impossible to judge the "purity" of a preparation from the measured specific biological activity only. In the second instance, for glycoproteins the answer to the first question may be extended as follows: a highly purified hormone preparation is one with a specific biological activity that is at least as high as the plateau-value known by previous experience. Although the results for

the specific activities of the LH preparations reveal variation (Table 2 - I), in general these values can be regarded to be of the same order as those reported in the literature. The biological activity of a hormone is determined by one or more active sites of the molecule. Changes in the amino acid sequence which do not involve the active sites, therefore, do not necessarily change the function. This situation is the cause of the existence of different molecular forms of the same hormone. Thus, a hormone preparation may contain many molecular forms differing as to their amino acid composition and, in the case of glycoproteins, also as to their carbohydrate composition, especially sialic acid content as mentioned earlier. The physico-chemical properties of such molecular forms, accordingly, also may show considerable variation, which would become visible when a hormone preparation is subjected to e.g. isoelectric focussing.

A so-called highly purified hormone preparation may turn out to be heterogeneous. This was found to be the case for the human LH preparations used in this investigation *(chapter 3)* as well as for LH preparations from several other species *(Reichert, 1971)*. When *Roos, Nyberg, Wide & Gemzell (1975)* submitted a highly purified human LH preparation to polyacrylamide electrophoresis, four components could be isolated and characterized. In a number of analyses these four components appeared to be homogeneous. Moreover, microheterogeneity beyond the limits of resolution cannot be excluded. In conclusion, the answer to the first question could be adjusted as follows: a highly purified hormone preparation is one that

does not show any heterogeneity.

If somebody would be succesful in obtaining an LH preparation that meets the criteria mentioned above, he then might wonder to what extent this preparation represents the *in vivo* circulating hormone. This is important to know when experiments using the LH preparation are to be interpreted for the *in vivo* situation. The traditional concept that the pituitary gland secretes one molecular form of each hormone must be abandoned. The notion of normal variability in LH is also supported by the results of a study by *Robertson, Van Damme & Diczfalusy (1975)*. From this investigation it may be inferred that a variety of LH forms exists in a single pituitary gland. There exists also a second type of variability. From studies in the monkey and the rat *(Bogdanove, Nolin & Campbell, 1975; Peckham, Yamaji, Dierschke & Knobil, 1973)* it may be concluded that the pituitary gland modulates i.e. secretes different molecular forms of a hormone in relation to the endocrine status of the body. These authors demonstrated a relationship between the behaviour of pituitary FSH on gelfiltration columns and the existing or induced endocrine condition of the animals. This last concept suggests that LH in prepubertal children may differ from that in people in the reproductive age which in turn may differ from that in aged people, but also that sex-linked differences may occur. In this respect, ICSH (interstitial cell-stimulating hormone) in the male and LH in the female might well be considered to be different molecular forms of the same hormone.

The same concept may also imply, that certain infertility syndromes can be carried back to deviations in the molecular hormone structure. Consider, for instance, the Klinefelter syndrome. The pituitary gland of patients suffering from this chromosomal disorder, secrete large amounts of LH (ICSH) that can be measured by *in vivo* bioassays as well as by RIA, but the testosterone production is impaired. The Leydig cell function can be restored, however, by injection of hCG *(Smals, Kloppenborg & Benraad, 1974)*. This indicates that the molecular form(s) of the patient's own LH (ICSH) are not suitable to pass the message to the Leydig cells.

This second type of variability, related to age or general endocrine condition, could well be responsible for the presence of multiple LH forms in purified preparations from pituitary glands. It is, however, difficult to avoid such variability, since the supply of glands is limited and often the exact sources are not known. Commonly, these glands are from elderly people, as was the case for our batches. A third variable is induced through the techniques themselves: preservation conditions as well as isolation and purification procedures. This is illustrated by the result of our own investigation showing differences between LH preparations obtained from acetone-dried and frozen material.

Furthermore, in comparing the hormone preparation with the *in vivo* circulating hormone also the peripheral metabolism must be taken into account. From recent reports in the literature on gelfiltration studies as well as from

observations in our own laboratory it might be inferred that in the blood the hormone is associated with proteins of various sizes *(Leidenberger, Graesslin, Scheel, Hess, Lichtenberg & Bettendorf, 1976; Graesslin, Leidenberger, Lichtenberg, Glisman, Hess, Czygan & Bettendorf, 1976; Lequin, 1976)*. Finally, it has been shown that also free hormone subunits circulate in the blood *(Benveniste, Bell, Koeppel & Rabinowitz, 1973; Prentice & Ryan, 1975; Hagen, McNatty & McNeilly, 1976)*. In summary, it may be concluded that it is not justified to measure blood hormone levels, (usually done by radioimmunoassay) in terms of a standard preparation purified from pituitary glands. According to the principle of comparing "like" with "like", instead of a pituitary preparation, a serum pool should be used *(Jeffcoate & Hutchinson, 1971; Bangham & Cotes, 1974)*. If this is not feasible one might consider using a standard preparation obtained by a purification procedure based on the activity in the radioimmunoassay instead of a procedure based on the activity in an *in vivo* bioassay. This is already practice for hormones for which no *in vivo* bioassay is known to exist, e.g. human placental lactogen, or for the subunits of the glycoprotein hormones.

From the foregoing it follows that for a complete elucidation of the effect of a hormone, including its mechanism of action, pertinent preparations should be characterized by as many assay methods as are available, in addition to the *in vivo* bioassay. Bearing this in mind and applying it to the human LH preparations of this investigation,

the second question (which other properties can be recognized?) may now be answered. Nowadays, at least three such additional methods are at one's disposal, namely *in vitro* bioassay, radioimmunoassay and radioligand assay. (Recently a new method, the cytochemical assay, has been developed and so far has been used successfully for the measurement of ACTH, TSH and gastrin; preliminary results indicate that it may also be applied for LH measurement *(Chayen, 1976)*. Since this method was not available to us we shall leave it out of this discussion) In the chapters 5 - 8 the results of these three methods as well as those of the *in vivo* bioassay have been presented. The LH preparations could be divided into four groups: a. LH_I from acetone-dried glands; b. LH_{II} from acetone-dried glands; c. LH_I from frozen glands; d. LH_{II} from frozen glands. Since each preparation was purified from a batch of several thousands of pituitary glands, the presence of multiple molecular forms in each preparation is possible. Because the batches themselves had been composed in a similar way (mainly of glands from elderly people) it is highly probable that the above-mentioned variability was the same in all batches. Therefore, it was felt permissible to combine the results of all preparations belonging to the same group. For convenience, the combined values for the potencies are summarized in Table 9 - I.

From these figures the following conclusions may be drawn:

(1) For all assay methods the preparations from frozen glands show lower values than the corresponding preparations from

Table 9 - I. Comparison of LH_I and LH_{II} potencies obtained by different assay methods.

		in vivo bioassay IU 1st IRP LH bio/mg	*in vitro* bioassay IU 1st IRP LH bio/mg	RIA IU 1st IRP LH imm/mg	RLA IU 1st IRP LH bio/mg
		(mean, 95% fiducial limits)			
LH_I	acetone-dried	7400 (6370-8710)	2620 (1920-3490)	1900* (1670-2210)	6200* (5700-6800)
LH_{II}	acetone-dried	5650 (4940-6480)	4190 (3060-5680)	3000* (2790-3300)	17400* (16300-19000)
LH_I	frozen	4280 (2830-6150)	2060 (1780-2640)	810* (750-890)	4800* (4200-5500)
LH_{II}	frozen	3830 (1480-6920)	3610 (2720-4880)	2040* (1460-2680)	12000* (10300-13200)

* indicates significant difference between LH_I and LH_{II} prepared from the same source.

acetone-dried glands. It is conceivable that the earlier mentioned plateau-value of the specific activities for the former is lower than that for the latter. However, it is also possible that the purification procedure used is not adequate to completely remove the relatively large amount of inert proteins that occur in frozen glands.

(2) LH_I and LH_{II} are different molecular entities. Their differences concerning *in vivo* and *in vitro* biological activity may not be significant, those revealed by the RIA and the RLA definitely are significant. This conclusion is in agreement with the results of the physico-chemical characterization, namely the large differences in pI-value

and K_{av}-value, and smaller differences in amino acid composition and sialic acid content.

The most important observation concerns the "discrepancy" between the potencies determined by the bioassay *(in vivo* and *in vitro)* on the one hand, and those assessed by the RIA and the RLA on the other hand. According to the former assays, LH_I and LH_{II} have the same potency, but judged by the latter methods, LH_{II} is more potent than LH_I. The question arises how this discrepancy might be explained. Bioassays measure biological *effects* of LH. In contrast, in RIA and RLA reactive *parts* of the hormone molecules are quantified: in the RIA these parts concern immunologically reactive sites and in the RLA they regard binding sites, i.e. parts responsible for binding the hormone to the cell membrane of the target tissue. Therefore, the fact that in the RIA as well as in the RLA, LH_{II} is more potent than LH_I indicates that LH_{II} has a greater affinity for the antibody and also for the membrane site than LH_I. The difference in affinity between LH_I and LH_{II} can also be deduced from the results of the experiments shown in Fig. 7 - 3 and 8 - 3. Irrespective of the type of LH used for labelling in these experiments, LH_{II} is more potent than LH_I.

This notion, naturally, is of particular significance with regard to the RLA as the binding of the hormones to the receptor is a necessary step in eliciting a biological response such as observed in the bioassays. That LH_I and LH_{II}, nevertheless, do not differ in the bioassays would seem to be contradictory and opposing the view of those who regard the

RLA as a bioassay *(Sharpe, Shomaneah, Ellwood, Hartog & Brown, 1975; Schlamowitz, Cronquist, Esfahani & Ward, 1976)*. While there is a lack of information about the kinetics of hormone binding (association and dissociation) and of the significance of affinity for the ultimate physiological response of the target cell, recent investigations yielded interesting pertinent information. *Channing & Safrin (1971)* suggested the possibility that 1 - 10% of the binding sites on the cell membrane are required for the activation of the physiological process, and that these were of very high affinity, while 90 - 99% of the binding sites serve as a storage depot. Similarly, *Catt & Dufau (1973)* studying the effect of hCG on the testis, showed that occupation of only a small part of the binding sites on the target was sufficient to elicit a maximal testosterone production. They introduced the term "spare receptors" to describe the unoccupied binding sites. Applying a similar reasoning to the LH bioassays used in this investigation leads to the hypothesis that to elicit a response (OAAD and cAMP) occupation of only a small fraction of the binding sites is sufficient and that, as a consequence, the suspected affinity difference between LH_I and LH_{II} is unnoticed, unless this difference is very large.

The above concept has its implications for clinical endocrinology. Since the amount of hormone in serum and urine samples as a rule is too low to be measured by a proper *in vivo* bioassay, routinely the RIA is applied (although some investigators have stept over to RLA). Recently, also *in vitro*

bioassay capable of handling clinical samples have been reported. The first results indeed indicate discrepancies between measurements by *in vitro* bioassay and by RIA *(Van Damme, Robertson & Diczfalusy, 1974; Dufau, Pock, Neubauer & Catt, 1976)*. Although in practice, the RIA has proved to be a useful tool in clinical endocrinology, the clinician should be aware of the possibility that the immunologically measured hormone levels do not necessarily reflect the patient's real (patho-)physiological endocrine status, which - as far as we know - only can be due to biologically active hormones of hormone fragments.

EPILOGUE

"It is not my intention to prove that until now I have been right, but to find out if I have been. Yes, we will make everything dubious and uncertain again. We shall not progress with seven-league boots but rather at a snail's pace. Whatever we find today should be erased tomorrow .... and be rewritten only after due reassessment. And should we find a hypothesis of ours to be correct we will regard it with particular suspicion."

(Bertolt Brecht, *Galileo Galilae*)

Luteinizing hormone was purified from human pituitary glands. The preparations were characterized by a number of parameters concerning structure and function.

In chapter 1 the backgrounds and motives for this investigation are reviewed.

The purification procedure for crude glycoprotein material from acetone-dried pituitary glands as well as from frozen glands is outlined in chapter 2. By a series of ion-exchange chromatography steps the bulk quantities of LH (coded LH_{II}), FSH and TSH were separated from each other. Yet, the FSH fraction still contained a substantial amount of LH. This type of LH (coded LH_I) could be isolated by further ion-exchange chromatography of the FSH-fraction. Both LH-fractions were then further purified by gelfiltration on Sephadex G 100 and lyophilized. The final preparations were only minimally contaminated by FSH and TSH as was shown by bioassay and radioimmunoassay determinations (chapters 5 and 7). The LH_I and LH_{II} preparations thus obtained from each batch of pituitary material were compared in the ensuing characterization studies.

In chapter 3 some physico-chemical properties are discussed. Microheterogeneity in all preparations was indicated by the finding of more than one band in isoelectric focussing studies. LH_I and LH_{II} appeared to differ as to their isoelectric point, sialic acid content, amino acid composition and molecular size. The contamination of each preparation by

free LH-subunits was determined. From the combined results it was concluded that LH_I and LH_{II} are different molecular entities.

Chapter 4 introduces the assay methods (which are described in more detail in the following chapters) as well as some theoretical considerations about the validity and reliability of these methods.

The *in vivo* bioassays are described in chapter 5. The LH preparations have been tested extensively in the ovarian ascorbic acid (OAAD) method. It was felt pertinent to carry out also a number of determinations with the seminal vesicle weight (SVW) test since it has been shown, at least for hCG, that acute (OAAD) and chronic (SVW) tests may yield different results. Both methods showed that for acetone-dried material LH_I is slightly more active than LH_{II}; for frozen material no differences could be observed between LH_I and LH_{II} preparations.

In chapter 6 the *in vitro* bioassay is described. To avoid the need of using enzymatic treatment for dispersion of the tissue, a cell type, which is by nature already in suspension, was selected, namely granulosa cells from immature pigs. LH-induced progesterone production was taken as an end-point. However, although the progesterone production was increased when LH was present in the medium, the dose-response curve was erratic. Thereupon, instead of granulosa cells, intact medium-sized porcine follicles were tried. Again, no quantitative relationship between the dose of LH and the progesterone production could be established. Therefore, the cAMP production was taken as end-point instead. Although this system could be

used to measure *in vitro* LH activity, it proved to be a rather imprecise method because of the difficulty to circumvent variation in maturity of the follicles. The results showed no significant difference in activity between LH_I and LH_{II} preparations.

The radioimmunoassay is discussed in chapter 7. To avoid possible interference of the other glycoprotein hormones on the basis of similarity of the α-subunits, the assay systems used employed an antiserum raised against highly purified LH_β. Besides the standard LH_β-system a quick LH_β-system was developed mainly as a tool in the purification procedure, for monitoring the effluent of the chromatography and gel-filtration columns. This quick system employs a higher concentration of tracer and antiserum and is carried out at 37°C. All preparations were tested in both LH_β-systems. The LH_I preparations showed consistently lower activities than the LH_{II} preparations. Preparations from frozen glands had a lower activity than those from acetone-dried glands. Several possible explanations were discussed. Comparison of the results of each preparation obtained in standard and quick system led to the interesting discovery that LH preparations in solution are unstable at 37°C and that some dissociation into subunits then occurs.

In chapter 8 the radioligand assay is dealt with. A homogenate of ovaries from pseudo-pregnant rats was used as binding tissue. Although this assay system was somewhat less precise than the RIA the results were essentially the same: the LH_I preparations were less active than the LH_{II} preparations

and the preparations from frozen glands were less active than those from acetone-dried glands.

The general discussion (chapter 9) is divided in two parts. The first part deals with the problem of heterogeneity of hormone preparations and the presence of various molecular forms. It was concluded that it is essential to characterize highly purified hormone preparations by as many methods as possible; this enables the investigator to rate the experimental results designed to elucidate the mechanism of action at their proper value. The second part concerns a comparison of the results of the four assay methods used for the determination of the various activities in the LH preparations. It was concluded that the functional assay methods (*in vivo* and *in vitro* bioassay) and the structural assay methods (RIA and RLA) measure different activities. The opinion of some investigators that the RLA is to be regarded as a bioassay, is contradicted.

Luteïniserend hormoon werd gezuiverd uit menselijke hypofysen. De verkregen preparaten werden gekarakteriseerd door middel van een aantal parameters betreffende structuur and functie.

In hoofdstuk 1 wordt de vraagstelling, die leidde tot dit onderzoek, geïntroduceerd.

De zuiveringsprocedure voor ruw glycoproteïnen materiaal afkomstig van aceton-gedroogde hypofysen en van bevroren hypofysen is beschreven in hoofdstuk 2. Door middel van een reeks chromatografie - stappen over ionenwisselaars konden het grootste deel van het LH (code LH_{II}), het FSH en het TSH van elkaar gescheiden worden. Toch bevatte de ruwe FSH-fractie nog een aanzienlijke hoeveelheid LH. Dit type LH (code LH_I) kon door middel van verdere chromatografie over een ionenwisselaar van het FSH gescheiden worden. Beide LH-fracties werden verder gezuiverd door middel van gelfiltratie over Sephadex G 100 en tenslotte drooggevroren. De eind-preparaten bevatten slechts minimale contaminaties van FSH en TSH zoals via bioassay resp. immunoassay kon worden aangetoond.(zie hoofdstukken 5 en 7). De verkregen LH_I en LH_{II} preparaten werden vergeleken in verdere karakteriseringsproeven.

In hoofdstuk 3 wordt een aantal fysisch-chemische eigenschappen behandeld. Het vóórkomen van microheterogeniteit in alle preparaten werd afgeleid uit de aanwezigheid van een aantal verschillende banden in isoelectrische focusseringsstudies. Tussen LH_I en LH_{II} bleken verschillen te bestaan wat betreft isoelectrisch punt, siaalzuurgehalte, aminozuursamen-

stelling en molecuulgrootte. De contaminatie van vrije LH-subunits in de preparaten werd ook bepaald. Alle resultaten tezamen leidden tot de conclusie dat LH_I en LH_{II} verschillende moleculaire eenheden zijn.

In hoofdstuk 4 worden de bepalingsmethoden geïntroduceerd, die in de volgende hoofdstukken uitgebreider besproken worden. Tevens worden theoretische aspecten over de waarde en betrouwbaarheid van deze methoden belicht.

De *in vivo* bioassays zijn beschreven in hoofdstuk 5. De LH preparaten zijn uitgebreid getest met behulp van de ovariele ascorbinezuur depletie (OAAD) methode. Een aantal LH-bepalingen werd ook uitgevoerd met de zaadblaasgewichtstoename (SVW) test, omdat was aangetoond, althans van hCG, dat in kortdurende (OAAD) en chronische (SVW) methoden verschillende uitkomsten gevonden worden. Met beide methoden werd gevonden dat LH_I van aceton-gedroogde hypofysen iets actiever is dan LH_{II}; tussen LH_I en LH_{II} preparaten van bevroren materiaal werden geen verschillen gevonden.

In hoofdstuk 6 wordt de *in vitro* bioassay beschreven. Om het gebruik van enzymen, nodig voor de dispersie van het weefsel, te vermijden werd een celtype uitgekozen dat van nature al in suspensie is, namelijk granulosacellen van immature varkens. De door de LH geïnduceerde progesteronproduktie werd als eindpunt genomen. Hoewel de progesteronproduktie toenam wanneer het medium LH bevatte, was de dosis-responsie curve zeer onregelmatig. Daarom werden, in plaats van granulosacellen, intacte middelgrote varkensfollikels geprobeerd. Opnieuw bleek er geen kwantitatief verband te bestaan tussen

de dosis LH en de progesteronproduktie. Daarom werd de cAMP-produktie gekozen. Met dit systeem bleek het mogelijk de *in vitro* LH-activiteit te meten maar de methode bleek weinig precies te zijn. De moeilijkheid was om de variatie in de rijpheid van de follikels te vermijden. Uit de resultaten bleek dat er geen significant verschil in activiteit bestond tussen LH_I en LH_{II} preparaten.

De radioimmunoassay wordt besproken in hoofdstuk 7. Om te voorkomen dat de andere glycoproteine hormonen op basis van de gelijkheid van de α-subunits zouden interfereren, werd in de assaysystemen gebruik gemaakt van een antiserum opgewekt tegen hooggezuiverd LH_β. Behalve het standaard LH_β-systeem werd een snel LH_β-systeem ontwikkeld in hoofdzaak als hulpmiddel bij de zuiveringsmethode, namelijk om het eluaat van de chromatografie en gelfiltratie kolommen snel te kunnen doormeten. In dit snelle systeem wordt een hogere concentratie tracer en antiserum gebruikt en de incubatie vindt plaats bij 37°C. Alle preparaten werden in beide LH_β-systemen bepaald. De LH_I preparaten vertoonden steeds een lagere activiteit dan de LH_{II} preparaten. Ook hadden preparaten gemaakt uit bevroren hypofysen een lagere activiteit dan die, gemaakt uit aceton-gedroogde hypofysen. Een paar mogelijke verklaringen hiervoor worden besproken. Bij het vergelijken van de resultaten van elk preparaat in het standaard en het snelle systeem werd de interessante ontdekking gedaan dat LH preparaten in oplossing onstabiel zijn bij hogere temperatuur (37°C) en dat er spontane dissociatie in subunits plaatsvindt.

In hoofdstuk 8 wordt de radioligand assay besproken. Een homogenaat van ovaria van pseudozwangere ratten werd gebruikt als bindend weefsel. Ofschoon deze methode iets minder precies is dan de RIA waren de resultaten vrijwel hetzelfde: de LH_I preparaten waren minder actief dan de LH_{II} preparaten en de preparaten bereid uit bevroren hypofysen waren minder actief dan die, bereid uit aceton-gedroogde hypofysen.

De algemene discussie (hoofdstuk 9) valt uiteen in twee stukken. In het eerste deel wordt de problematiek van de heterogeniteit van hormoonpreparaten en de aanwezigheid van talrijke moleculaire vormen besproken. Geconcludeerd werd dat het van belang is om de hooggezuiverde hormoonpreparaten te karakteriseren met behulp van zoveel mogelijk methoden; dit geeft de onderzoeker een beter inzicht hoe de experimentele resultaten bij de opheldering van het werkingsmechanisme gewaardeerd moeten worden. In het tweede deel worden de resultaten besproken van de vier bepalingssystemen die gebruikt zijn om de verschillende activiteiten van de LH preparaten te meten. Als conclusie komt naar voren dat de "functionele" bepalingsmethoden (*in vivo* en *in vitro* bioassay) en de "structurele" (RIA en RLA) verschillende dingen meten, hetwelk in tegenspraak is met de mening van diegenen die de RLA beschouwen als een bioassay.

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CURRICULUM VITAE

De auteur van dit proefschrift werd in 1948 te Bandung geboren. Na het eindexamen gymnasium-β in 1965 behaald te hebben volgde hij aan de Brighton Polytechnic te Brighton, Engeland, het eerste jaar van de opleiding tot B.Sc. in Applied Chemistry. In 1966 werd gestart met de scheikunde studie aan de Universiteit van Amsterdam. In 1969 werd het kandidaatsexamen behaald en in 1971 het doctoraalexamen, met als hoofdvak biochemie, als bijvak dierfysiologie en als keuzevak klinische chemie. Van januari 1972 tot juli 1973 was hij werkzaam als wetenschappelijk medewerker aan de Universiteitskliniek voor Inwendige Ziekten in het Binnengasthuis te Amsterdam. Sinds juli 1973 is hij, in dienst van Z.W.O., verbonden aan de Universiteitskliniek voor Verloskunde en Gynaecologie in het Sint Radboudziekenhuis te Nijmegen.

STELLINGEN

Bij het onderzoek naar het werkingsmechanisme van eiwithormonen, dient men bij de interpretatie van de resultaten rekening te houden met heterogeniteit van de gebruikte "hoog gezuiverde" preparaten.
(Dit proefschrift)

Ten onrechte wordt door sommige onderzoekers de radioligand assay beschouwd als een bioassay.
(Schlamowitz M., Cronquist J., Esfahani M. & Ward D.N. (1976) Acta Endocr. (Kbh) 81, 270; Sharpe R.M., Shamanesh M., Ellwood M.G., Hartog M. & Brown P.S. (1975) J. Endocr. 65, 265)

De door de IUPAC-Commission on Biochemical Nomenclature aanbevolen namen voor peptide hormonen dragen, in tegenstelling tot het beoogde doel, niet bij tot duidelijkheid in de naamgeving.
(The nomenclature of peptide hormones (1975) J. Biol. Chem. 250, 3215)

Om te voorkomen, dat voor de in de toekomst vast te stellen internationale standaarden en referentie-preparaten de bestaande verwarring met betrekking tot de eenheden van biologische en immunologische activiteit voortduurt, dient de WHO Expert Committee on Biological Standardization terug te komen op haar besluit om deze twee soorten eenheden in getalswaarde zoveel mogelijk aan elkaar gelijk te kiezen.
(WHO - ECBS 21th Report (1969) WHO Techn. Rep. Ser. 413, 8)

Het na de dood afstaan van organen ten behoeve van transplantatie of isolatie van anderszins niet te verkrijgen componenten moet beschouwd worden als een plicht jegens de medemens. De wettelijke regeling op dit punt dient verbeterd te worden.
(C.F. Wery, Beschikken over eigen lichaam. Ars Aequi XXV p. 305)

Het vermelden van de activiteit van een testis carcinoom in hoeveelheden humaan chorion gonadotropine (hCG) per ml serum is onjuist omdat hCG per definitie afkomstig is van placentair trofoblastweefsel.

Door de van oudsher autoritaire positie en de daaruit voortvloeiende mentale instelling van de arts is er een gerede kans dat de relatie clinicus - niet clinicus, bij het bepalen van het gezamenlijk te voeren beleid in multidisciplinaire onderzoeksprojecten, negatief beïnvloed wordt.

Artikel 23 van de Promotieregeling vormt een inbreuk op de vrijheid van meningsuiting; deze inbreuk is slechts mogelijk op grond van het feit dat de Katholieke Universiteit Nijmegen formeel geen overheidsinstelling maar een stichting is.
(P.J. Boukema, Enkele aspecten van de vrijheid van meningsuiting in de Duitse Bondsrepubliek en in Nederland. Alphen a/d Rijn (1967) p. 219 ev)

Voor het efficient verrichten van wetenschappelijk onderzoek is een goed georganiseerde wetenschappelijke bibliotheek onmisbaar.

De gynaecoloog, die in het kader van de diagnostiek van infertiliteit een pakket van onderzoekingen opstelt, realiseert zich vaak onvoldoende in welke mate de inspanningen, die de echtelieden zich in deze moeten getroosten, een verstoring van de sexuele relatie ten gevolge kunnen hebben.

Teneinde een werkelijke belangenbehartiging van de burgers te waarborgen, verdient het aanbeveling dat de overheid bij het uitvoeren van openbare werken de Wet op de Ruimtelijke Ordening met grotere zorgvuldigheid in acht neemt.

Gezien de talrijke problemen van psychische en fysieke aard, die de voortplanting bij de *mens* kan veroorzaken, moeten de *kabouters* beschouwd worden als fortuinlijke wezens.
(W. Huygen en R. Poortvliet, Leven en werken van de kabouter, Van Holkema & Warendorf, Bussum)

De polemiek, die pleegt te ontstaan na een Oudejaarsavond-conférence van Wim Kan, getuigt van een gebrek aan gevoel voor humor bij het Nederlandse volk.

J.G. Loeber
22 april 1977